Assessment of *Moringa oleifera* and *Xylopia aethiopica* extracts for their preservative potential in fruit juice

by

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Declaration

I, Gloria Onyeka Baah, do hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree Master of Health Sciences in Environmental Health, is my own independent work; and it complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself of any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

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Signature of student **Date**

Dedication

I dedicate this work to my heavenly Father, family and everyone who contributed to the success of this study.

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My sincere gratitude goes to:

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Summary

The chemical compounds used as preservatives in fruit juices have been associated with many health problems. Hence, it is imperative to find safer alternatives from natural sources such as plants. This study assessed *Moringa oleifera* and *Xylopia aethiopica* extracts for their preservative potential in fruit juice based on their phytochemical contents, antioxidant activities and anti-yeast properties. Qualitative phytochemical analysis showed that *M. oleifera* extracts contained alkaloids, tannins, saponins, glycosides, terpenoids and flavonoids but the water extracts lacked alkaloids. The same phytochemicals, except terpenoids, were also found in all *X. aethiopica* extracts. The antioxidant activity determined using 2, 2- dipheny- 1-picrylhydrazyl (DPPH) and the total phenolic content expressed as Gallic Acid Eqivalent (GAE/g), indicated that the plant extract with the highest antioxidant activity (IC_{50}) also had the highest total phenolic content (GAE/g). The methanol extract of *M. oleifera* had IC_{50} of 48.09 µg/mL and GAE/g of 110.0 mg while the acetone extract of *X. aethiopica* had IC_{50} of 19.10 µg/mL and GAE/g of 173.2 mg. The agar dilution assay was used to evaluate the anti-yeast activity of the extracts *in vitro* against fruit juice spoilage yeasts. The methanol extract of *M. oleifera* had the strongest anti-yeast activity and was specifically fungicidal against the CY0757 and IGC4242 strains of *Zygosaccharomyces bailii* and *Wickerhamomyces anomalus*. The acetone and water extract were poor growth inhibitors. All *M. oleifera* extracts showed stimulatory activity on *Yarrowia lipolytica* which has been reported as beneficial in biofuel production. The best anti-yeast activity for *X. aethiopica* was shown by the methanol extract which was fungicidal against *Zygosaccharomyces bailii* IGC4242 and *Cryptococcus laurentii*. The acetone extract of *X. aethiopica* exerted inhibitory activity against most of the yeasts. The water extract showed the least bioactivity. The anti-yeast activity of plant extracts in fresh grape and orange juice showed microbial overgrowth in the untreated fruit juices used as controls, indicating the presence of contaminants in treated fruit juices. The Hoechst 33342 and propidium iodide dual staining procedure showed that *M. oleifera* extracts were not cytotoxic on normal Vero cells. The water extract of *X. aethiopica* also did not show toxicity on the cells. The methanol and acetone extract were, however, very

toxic on the cells. This study demonstrates that although *M. oleifera* and *X. aethiopica* extracts have limited potential as natural preservatives in fruit juice, the growth stimulatory activity of *M. oleifera* extracts on *Y. lipolytica* may be useful in biofuel production and this requires further investigation.

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List of abbreviations

%	Percentage
µg/mL	Microgram per millilitre
ADI	Acceptable daily intake
Anova	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAFSaB	Centre for Applied Food Sustainability and Biotechnology
CDC	Centre for Disease Control and Prevention
cm	Centimetre
CO_2	Carbon dioxide
Cu^{2+}	Copper ion
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
DPPH	2, 2- dipheny- 1- picrylhydrazyl
EFSA	European Food Safety Authority
FAO	Food and Agricultural Organisation
FBS	Foetal Bovine Serum
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FSAI	Food Safety Authority of Ireland

g/L	Gram per Litre
GAE/g	Gallic acid equivalent per gram
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
HNO_2	Nitrous acid
IC_{50}	The concentration of sample that gives 50% of maximum response
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LSD	Least Significance Difference test
mg/mL	Milligram per millilitre
MIC	Minimum inhibitory concentration
mL	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
$NaCO_3$	Sodium carbonate
NO_2	Nitrites
NO_3	Nitrates
NOC	N-nitroso compounds
^oC	Degree Celsius
PDA	Potato Dextrose Agar
PG	Propyl gallate

PI	Propidium iodide
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Standard deviation
TBHQ	Tertiary butyl hydroquinone
USFDA	United States Food and Drug Administration
WHO	World Health Organisation
YPD	Yeast extract Peptone Dextrose

Chapter One

Introduction

Food spoilage occurs when the organoleptic and nutritional qualities of food are altered such that it becomes undesirable or unfit for human consumption (Rawat et al., 2015). Food spoilage results in waste (Figure 1.1) and may occur at any time from the moment of harvesting to processing. This food spoilage may be caused by physical damage during storage or transportation, insect pest activities, the reaction of chemical compounds in food e.g. oxidation and microbial contamination with protozoa, yeasts, moulds or bacteria (Rawat et al., 2015).

Microbial contaminations account for about 25% of post-harvest food supply losses (Snyder and Worobo, 2018). Food spoilage caused by microbial contaminations is a serious threat to public health, food industries and the environment worldwide (Penha et al., 2017). In fact, thousands of people, especially the elderly, children, pregnant women and people with compromised immune systems get ill and many lose their lives as a result of exposure to contaminated food or water. The Centre for Disease Control and Prevention (CDC) estimates that every year around 48 million people fall ill and 3000 die due to foodborne diseases (CDC, 2015; Penha et al., 2017).

Figure 1.1. Food wastage due to spoilage (FAO, 2019)

Beyond the negative impact on health, microbial food spoilage causes huge economic losses in the food industry due to product recalls that invariably lead to negative brand image and reduction in sales (Biango-Daniels et al., 2019). According to the Food and Agricultural Organisation (FAO), about one third of food (approximately 1.3 billion tonnes) produced globally is wasted due to spoilage and this amounts to a loss of roughly US$ 680 billion for industrialised countries and US$ 310 for developing countries (FAO, 2019). Similarly, food spoilage results in waste of resources such as land, capital, water and labour, culminating in environmental harm in the form of excessive greenhouse gas emissions that contribute to climate change (FAO, 2018).

Fresh produce, including roots, tubers, fruits, vegetables (Figure 1.1) and products derived from fruits and vegetables e.g. juices are prone to spoilage largely because of their compositions. For example, enzymatic reactions that occur during the ripening stage of fruits and vegetables make them softer in texture and easily accessible to microorganisms. Also, during fruit processing, fresh juices come in contact with air and microorganisms in the environment, leaving them exposed to spoilage (Rawat et al., 2015; Snyder and Worobo, 2018).

Fruit juices are tasty and widely consumed beverages rich in vitamins, minerals and bioactive compounds with many advantages for human health. Fruit juices have low pH values that many microorganisms find unsuitable for growth; however, a variety of microorganisms such as acid tolerant bacteria, yeasts and moulds can thrive in fruit juices and cause spoilage (Ephrem et al., 2018).

Among microorganisms, yeasts are the main agents of spoilage in fruit juices because many of them can tolerate high acidic conditions and thrive in the absence of oxygen. Fruit juice spoilage due to yeast culminates in the loss of nutritional and sensory qualities, waste, health challenges and economic losses (Bukvicki et al., 2014; Kaczmarek et al., 2019). Owing to these challenges, different methods such as pasteurisation and the use of chemical compounds e.g. sulphur dioxide, benzoic

and sorbic acids are traditionally applied in the preservation of fruit juice (Guedes et al., 2016).

1.1. Rationale

Although traditional methods of preserving fruit juices (pasteurisation and the use of chemical compounds) are effective, they come with several disadvantages. Fruit juices that have undergone pasteurisation lose their nutritional and sensory qualities and most chemical compounds used in fruit juice preservation have been reported to pose various risks to human health (Guedes et al., 2016). Even so, their use in the fruit juice industry is still pervasive. Consequently, it is important to find safer, effective and environmentally friendly alternatives from natural sources that could be used in place of these conventional preservatives.

Various spices and herbs such as *Cinnamomum zeylanicum* (cinnamon), *Zingiber officinale* (ginger), *Syzygium aromaticum* (cloves), *Xylopia aethiopica* (negro pepper), *Ocimum basilicum* (basil), *Salvia officinalis* (sage), *Moringa oleifera* (ben oil tree), *Rosmarinus officinalis* (rosemary) and many others contain phytochemicals with antioxidant and antimicrobial properties, thus making them potentially rich sources of novel biostatics, biocides and preservatives. Based on previous reports that extracts from *Moringa oleifera* and *Xylopia aethiopica* have good antioxidant and antimicrobial properties (Adefegha et al., 2018; Liu et al., 2018), it is necessary to explore their potential for use as fruit juice preservatives.

1.2. Aim of the study

The aim of this study was to assess *Moringa oleifera* and *Xylopia aethiopica* extracts for their preservative potential in fruit juice.

1.3. Objectives of the study

i. To optimise *M. oleifera* and *X. aethiopica* extracts for fruit juice preservation.
ii. To determine the phytochemical constituents, antioxidant activity and total phenolic contents of the plant extracts.

iii. To evaluate the *in vitro* anti-yeast activity of *M. oleifera* and *X. aethiopica*.

iv. To investigate the effect of the plant extracts on fresh grape and orange juice.

v. To evaluate the *in vitro* cytotoxicity of the plant extracts used.

In order to fulfil the study objectives, a five-step procedure was used as shown in Figure 1.2.

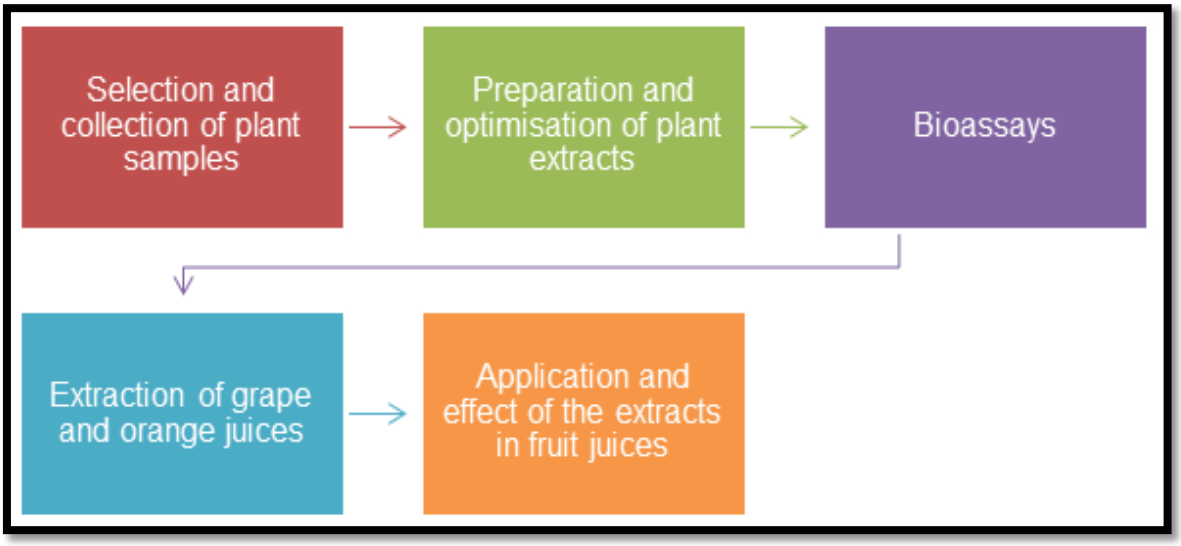

Figure 1.2. Flow chart of the procedure used to investigate the preservative potential of *M. oleifera* and *X. aethiopica* extracts in fresh grape and orange juice

1.4. Dissertation layout

This study is structured in six chapters. Chapter one gives a general introduction and outlines the rationale, aims, objectives and layout of the study. Chapter two reviews the literature on fruits and fruit juices (grapes and oranges), their importance in health, methods of preservation and drawbacks as well as the potential of *M. oleifera* and *X. aethiopica* as natural preservatives. The third Chapter concentrates on the phytochemical constituents, antioxidant activity and total phenolic content of 50% methanol, 50% acetone and water extracts of *M. oleifera* leaves and *X. aethiopica* fruits. The *in vitro* anti-yeast activity of the extracts from *M. oleifera* and *X. aethiopica* are presented in Chapter four. The application and assessment of the effects of the extracts in fresh grape and orange juice are an integral component of

this chapter. The penultimate Chapter five discusses the *in vitro* cytotoxicity of extracts from *M. oleifera* leaves and *X. aethiopica* fruits. Chapter six of this dissertation presents a general discussion, conclusions and recommendations from the study.

1.5. References

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Biango-Daniels, M.N., Snyder, A.B., Worobo, R.W. and Hodge, K.T. 2019. Fruit infected with *Paecilomyces niveus*: A source of spoilage inoculum and patulin in apple juice concentrate? *Food Control,* 97: 81-86.

Bukvicki, D., Gottardi, D., Tyagi, A.K., Veljic, M., Marin, P.D., Vujisic, L., Guerzoni, M.E. and Vannini, L. 2014. *Scapania nemorea* liverwort extracts: Investigation on volatile compounds, *in vitro* antimicrobial activity and control of *Saccharomyces cerevisiae* in fruit juice. *LWT - Food Science and Technology,* 55: 452-458.

Centre for Disease Control and Prevention (CDC). 2015. Vital Signs. Available online at https://www.cdc.gov/VitalSigns/foodsafety/ Accessed on the 24th of January, 2020.

Ephrem, E., Najjar, A., Charcosset, C. and Gerges, H.G. 2018. Encapsulation of natural active compounds, enzymes, and probiotics for fruit juice fortification, preservation, and processing: An overview. *Journal of Functional Foods*, 48: 65-84.

Food and Agriculture Organisation (FAO) of the United Nations. 2018. Food losses and waste: Issues and policy options- global perspectives studies. Available online at http://www.fao.org/3/ca1431en/CA1431EN.pdf Accessed on the 24th of January, 2020.

Food and Agriculture Organisation (FAO) of the United Nations. 2019. Save Food: Global initiative on food loss and waste reduction. Available online at http://www.fao.org/save-food/resources/keyfindings/en/ Accessed on the 6th of April, 2019.

Guedes, J.P.S., da Costa Medeiros, J.A., de Souza e Silva, R.S., de Sousa, J.M.B., da Conceição, M.L. and de Souza, E.L. 2016. The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices. *International Journal of Food Microbiology,* 238: 183-192.

Kaczmarek, M., Avery, S.V. and Singleton, I. 2019. Microbes associated with fresh produce: Sources, types and methods to reduce spoilage and contamination. *Advances in Applied Microbiology*, 107: 29-82.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

Penha, C.B., Bonin, E., da Silva, A.F., Hioka, N., Zanqueta, E.B., Nakamura, T.U., Filho, B.A.A., Campanerut-Sa, P.A.Z. and Mikcha, J.M.G. 2017. Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin. LWT - *Food Science and Technology,* 76: 198-202.

Rawat, S. 2015. Food Spoilage: microorganisms and their prevention. *Asian Journal of Plant Science and Research*, 5(4): 47-56.

Snyder, A.B. and Worobo, R.W. 2018. The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers. *Food Control,* 85: 144-150.

Chapter Two

Literature review

2.1. Introduction

A fruit is the seed-bearing structure in angiosperms (flowering plants) formed from the ovary after flowering. In angiosperms, fruits play a major role in the propagation of seeds for the continued existence of the species. From time immemorial, fruits have served as important sources of food for humans and animals creating a symbiotic relationship for seed dispersal (Cole et al., 2019).

Fruits have earned a unique spot in dietary guidance due to their richness in sugars (glucose, fructose and sucrose), vitamins, minerals, dietary fibres, antioxidants and bioactive compounds which are all essential in the human diet. Fruits can be categorized into dry fruits e.g. cereals, grains, nuts, etc. and fleshy fruits e.g. apples, mangos, guavas, pears, bananas, pineapples, oranges, grapes and so on (Oluwole et al., 2016).

2.2. Sweet orange (*Citrus sinensis*)

The genus *Citrus* of the Rutaceae family is the most significant fruit crop cultivated worldwide with an annual yield of approximately 124 million tonnes in 2016 (FAO, 2017). Different species of *Citrus* include *C. limon* (lemon), *C. reticulata* (mandarin, tangerine), *C. paradisi* (grapefruit), *C. clementina* (clementine), *C. aurantium* (sour orange) and *C. sinensis* (sweet orange) (Favela-Hernández et al., 2016).

Citrus sinensis (Figure 2.1) is a small evergreen flowering tree of about 9 -10 m in height with big thorns on branches. The leaves are 4 - 10 cm long, oval shaped and alternately arranged. The fruit may be spherical or oval in shape and consists of two different parts; the endocarp (pulp with juice sac) and the pericarp (peel) which changes from green to orange or yellow colour when ripe (Etebu and Nwauzoma, 2014).

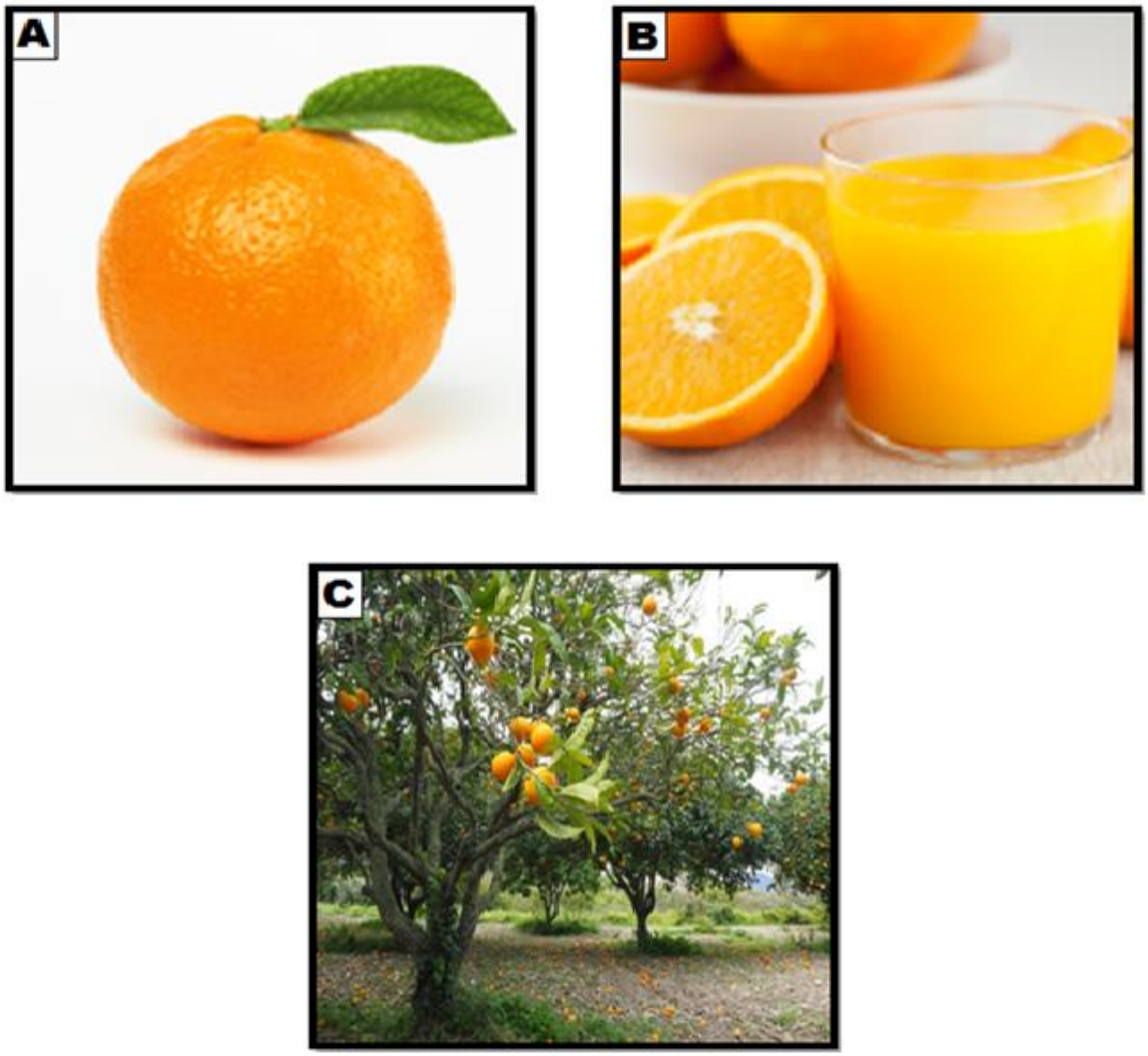

Figure 2.1. (A) Ripe orange with leaf, (B) Half-cut orange and orange juice, (C) An orange tree bearing fruits (Florida Department of Citrus, 2012).

Citrus sinensis constitutes about 70% of the entire annual yield of citrus species which makes it the largest citrus species grown across the globe. Although sweet orange is indigenous to Asia, it is widely cultivated in tropical and subtropical areas of the world (Favela-Hernández et al., 2016). According to the Food and Agricultural Organisation (FAO, 2017), the major five producers of sweet oranges are Brazil, China, India, Mexico and USA. In addition to the immense economic value of sweet oranges and juices, essential oils derived from sweet oranges are exploited by the cosmetic, food and beverage industries worldwide (Fatta Del Bosco et al., 2020).

Apart from the delicious taste of sweet orange, it is rich in different minerals, vitamins (especially vitamin C, a strong antioxidant that helps in boosting the body's immune system) and phytochemicals. The abundance of phytochemicals and fibre in sweet orange makes it a useful remedy for various health conditions like constipation, diarrhoea, menstrual disorder, depression, obesity, hypertension, anxiety, cough, cramps, cold, scurvy, stress and many other ailments (Mannucci et al., 2018).

The sweet orange peel contains a microflora that mostly comprises bacteria and fungi (yeasts and moulds), some of which may be unintentionally introduced into fruit products during processing and cause spoilage. Moreover, raw fruits may contain pathogens such as *Campylobacter* spp., *Clostridium botulinum, Salmonella* spp., *Listeria monocytogenes, Escherichia coli* and *Bacillus cereus* that can cause sicknesses in humans; this makes thorough washing of the fruit a necessity before direct consumption, extraction of juice or essential oils (Etebu and Nwauzoma, 2014; Al-Kharousi et al., 2016).

2.3. Grapes (*Vitis vinifera*)

Grapes (*Vitis* spp.) are woody vines that belong to the Vitaceae family. They are important fruits widely cultivated and consumed across the world (Gomes et al., 2019). The total global production for grapes in 2016 was approximately 75 million tonnes with countries like China, Italy, USA, Spain and France being the top five producers (FAO, 2017). Grapevines (*Vitis*) consist of various species such as *V. rotundifolia, V. labrusca, V. aestivalis, V. amurensis, V. californica, V. cinerea, V. girdiana, V. riparia, V. vinifera* and many others (Everhart, 2010).

Vitis vinifera is a liana (climbing plant) that grows by winding its tendrils around trees, fences and trellis, for support (Figure 2.2). This liana can grow up to 35 m in height. The leaves are large and orbicular in shape. The fruits are round or oval shaped and may be red, green or purple in colour (Everhart, 2010; DAFF, 2012). Peel, pulp and seeds constitute the grape berry although this composition depends

greatly upon the grape species, variety, cultivation, climate and processing factors (Gomes et al., 2019). Grape berries grow on flower clusters and during their developmental and ripening phases they undergo a series of physical and molecular changes such as colour development, softening, production of volatiles, acid catabolism and sugar accumulation which, upon maturation, give off appealing signals to humans, birds and other vectors involved with seed dispersion (Ghan et al., 2017).

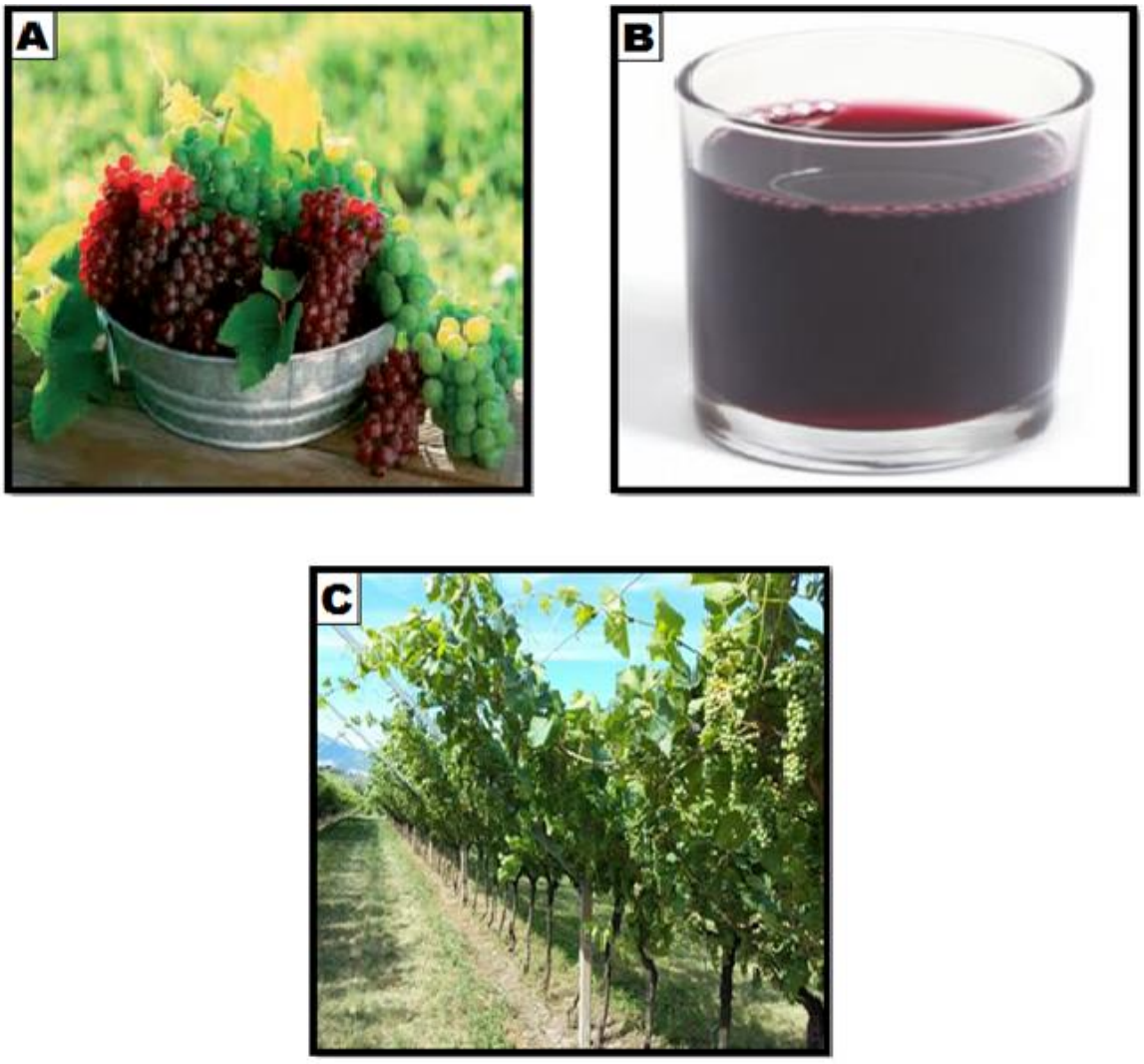

Figure 2.2. (A) A bowl of white and red grapes with leaves, (B) Red grape juice, (C) Grape berries on a tree branch (DAFF, 2012).

Grapes can either be eaten raw (fresh or dried) or after being processed into other forms like jelly, juice, jam and wine. Grapes and grape products have many nutritional and bioactive properties that are beneficial to human health. They are rich sources of polyphenols and carotenoids. Not only has the consumption of fresh grapes increased recently, but the interest in grape derivatives, specifically grape juices, have also been on the rise because they offer a diversity of phytochemicals with many health benefits (DAFF, 2012; Gomes et al., 2019).

2.4. Fruit juices

Fresh fruits are seasonal and highly deteriorative in nature which makes processing them into fruit juices a necessity so that they can be readily available to consumers during off-season (Oluwole et al., 2016). Fruit juice is a non-sparkling, unfermented but fermentable liquid extracted from one or more fruits. This ready-to-serve beverage is derived either from the mechanical extraction of juice from healthy and mature fruits or by reconstituting concentrated fruit juice, pulp or puree with pure water (Oluwole et al., 2016). Fruit juices are richly packed with essential vitamins and minerals and contain almost the same nutrients as found in the fruits from which they are obtained (IFU, 2017).

The fruit juice industry is one of the major sectors in the beverage industry worldwide. Over the years, the consumption of fruit juice has increased significantly as fruit juices have now become essential ingredients of contemporary diets in many parts of the world owing to their broad beneficial health effects. When fruit juices are processed hygienically, they provide many health promoting properties like improving the body's immune system, promoting strong bones and teeth, supporting digestion, reducing the occurrences of different chronic and degenerative diseases such as congestive heart failure, cancers, diabetes and stroke (Nayik et al., 2013; Pascual-Ahuir et al., 2015).

Orange juice is a tasty and nutritious drink derived from the endocarp of the *C. sinensis* fruit. The leading varieties from which orange juice is produced are Hamlin,

Pineapple, Valencia and Pera (Chanson-Rolle et al., 2016). It is a beverage widely consumed across the world for its rich taste and various health benefits e.g. lowering the risks of various diseases and enhancing general wellbeing. Orange juice has an abundance of sugars (sucrose, glucose and fructose), amino acids, minerals, vitamins, antioxidants and a variety of other bioactive compounds (Goulas and Manganaris, 2012; Anvarian et al., 2018).

Grape juices are produced from any mature and sound grape variety. *Vitis labrusca* L. varieties, for example, concord and Isabel are the main cultivars used in making grape juice because they produce a very nutritious and aromatic juice of great quality (Dal Magro et al., 2016). Grape juice is an excellent source of polyphenols, vitamins and minerals. It has been reported that ingesting pure 100% grape juice could reduce the risks of cardiovascular disease, diabetes mellitus and hypertension (Hyson, 2016).

2.5. Food spoilage

Food spoilage is a metabolic process that causes undesirable alterations in food, making it unpalatable or unsuitable for human consumption. Some spoiled foods which do not contain pathogens or toxins that induce sickness in consumers may be considered safe to eat. However, when food spoils unpleasant changes occur in the sensory characteristics such as texture, odour, taste and general appearance, making it unappealing to humans (Rawat, 2015). These changes can be classified into three categories;

i. Chemical food spoilage: this occurs from the reaction of different constituents in food; e.g. oxidation, enzymatic browning and non-enzymatic browning.

ii. Physical food spoilage: this type of spoilage is as a result of either moist food becoming extremely dehydrated or dry foods becoming exposed to excess moisture.

iii. Microbiological spoilage: the growth of microorganisms in food brings about the production of enzymes and by-products that result in microbiological spoilage (Benner, 2014; Petruzzi et al., 2017).

An appraisal by the Food and Agricultural Organisation (FAO) indicates that approximately 45% of fresh produce, processed fruits and vegetable go to waste due to their high perishability. Food spoilage caused by microbial contamination has been a very big problem in the food industry. Apart from customers discontent with the associated brand which may lead to reduction in sales and job cuts (FAO, 2012; Snyder and Worobo, 2018), microbial contaminations of food may bring about enormous health consequences and economic losses due to product recall and waste (Park et al., 2019). For example, PepsiCo, a multinational food and beverage company recalled a batch of one of its products called Tropicana Trop 50 multivitamins juice in 2018, due to spoilage attributed to progressive fermentation of the juice. Customers were advised against drinking any one litre juice with characteristic fizziness, off-taste, sediments or bloated bottles and to return the products for a full refund (FSAI, 2018).

Freshly squeezed and unpasteurized fruit juices are, however, more prone to spoilage because their protective layer which is the skin or cell wall, is destroyed during juice production thereby exposing the fluid to air and the surrounding microorganisms (Aneja et al., 2014). The presence of microorganisms in fruit juices may arise from poor hygiene during handling and processing or improper washing of the fruits during preparation because of the microflora of raw fruits (Oluwole, 2016). No less than 48 cases of fruit juice contamination were recorded between 1974 and 2014. Most of the contamination outbreaks were caused by consuming fresh and unpasteurized orange and apple juice fouled with pathogenic bacteria (*Shigella flexneri*, *Salmonella* spp., enterotoxigenic *Escherichia coli* and Shiga toxin-producing *Escherichia coli* O157 and O111 STEC), viruses (norovirus genotype II and hepatitis A virus) or protozoa (*Cryptosporidium* and *Trypanosoma cruzi*). *T. cruzi* in particular

accounted for outbreaks that resulted from the consumption of fresh juice obtained from different exotic fruits (Martínez-Gonzáles and Castillo, 2016).

In order to reduce the risks of outbreaks, a law regarding the processing of fruit and vegetable juices was passed in 2001 (Juice Hazard Analysis and Critical Control Point) by the United States Food and Drug Administration (USFDA). The law warranted a ≥ 5-log reduction (99.999%) of the pathogens of concern in juices; although no specific method was recommended for use (USFDA, 2001).

Microbial contamination of fruit juices is determined by various factors such as the wholesomeness of fruits, quality of water (which can be a major source of contamination), equipment and other raw materials (sugar and concentrate syrups) used during preparation. Extracting juice from unhealthy fruits such as those that are rotted, bruised or picked from the ground are likely sources of juice contamination. Such fruits can harbour pathogenic organisms that bring about sicknesses in humans. Also, during the preparation of fruit juices, microorganisms from unsterile water, utensils and raw materials may be unwittingly passed into fruit juices and cause spoilage (Nayik et al., 2013; Martínez-Gonzáles and Castillo, 2016). In addition, airflow in industrial facilities could be another major source of contamination of fruit juice. Microorganisms are very small in size and can attach to dust particles present in air which facilitates their dispersion and thereby increasing the risks of microbial contamination in fruit juices (Hernández et al., 2018).

Various microorganisms e.g. acid tolerant bacteria (*Zymomonas, Alicyclobacillus, Gluconobacter, Acetobacter, Lactobacillus, Leuconostoc, Zymobacter* and *Bacillus*), yeasts (*Saccharomyces, Rhodotorula, Pichia, Candida, Zygosaccharomyces,* etc.) and moulds (*Penicillium, Cladosporium, Aureobasidium, Aspergillus* and *Botrytis*) have been isolated from spoiled fruit juices (Aneja et al., 2014); however, yeasts whose growth are favoured by the low pH, high sugar levels and other nutritional properties of fruit juices (Maciel et al., 2013) are further discussed in this study.

2.6. Yeast spoilage in fruit juice

Yeasts are eukaryotic, unicellular organisms that belong to the Fungi domain. They are specifically the main spoilage organisms in fresh and concentrated fruit juices with cfu/mL ranging from <1.0 to 6.83 log10 (Mahgoub and El-Shourbagy, 2015) because they can tolerate high acidic conditions and quite a number of them can grow anaerobically (Bukvicki et al., 2014). The genera mostly associated with fruit juice spoilage are *Candida, Rhodotorula, Saccharomyces* and *Pichia* while the species commonly present in spoiled citrus juices are *Saccharomyces cerevisiae, Torulaspora delbrueckii, Zygosaccharomyces rouxii, Candida stellata* and *Candida parapsilosis* (Aneja et al., 2014). Yeast spoilage usually results in off-flavours, off-odour, flocculation, carbon dioxide and alcohol production, turbidity and phase separation through the action of enzymes on the pectin (Maciel et al., 2013; Bukvicki et al., 2014).

Particularly, thermophilic and acid-tolerant yeasts from the *Zygosaccharomyces* genus remain a major threat to the fruit juice industry. *Zygosaccharomyces* are extreme osmotolerant organisms with peculiar characteristics such as the ability to resist weak acid preservatives, ferment hexose sugars and thrive at low pH. *Zygosaccharomyces bailii, Zygosaccharomyces rouxii* and *Zygosaccharomyces lentus* are the three most frequently isolated species from spoiled fruit juices (Wang et al., 2016). Spoilage caused by these organisms is often evident as growth which can be easily seen on the surface of the juice or fermentative spoilage such as off-odours, off-flavours and excess carbon dioxide production which may result in the swelling and bursting of juice containers or bottles (Wang et al., 2016). In view of this, different preservative methods have been employed in order to tackle the challenge of microbial spoilage in fruit juices.

2.7. Food preservatives

Food preservatives are natural or chemical substances deliberately incorporated into food or food products to impede spoilage caused by microbial contamination thereby prolonging the shelf life and maintaining the quality and safety of the food. The main

aim of food preservatives is to prevent the growth of spoilage and pathogenic microorganisms while preserving the nutritional and sensory characteristics of food. These are generally accomplished by removing microorganisms, air and moisture from food or making the food environment unsuitable for spoilage and pathogenic microorganisms to thrive (Abdulmumeen et al., 2012; Singh et al., 2019).

Historically, drying and smoking were popular methods for preserving foods such as fruits, vegetables, fishes and meats because most bacteria and fungi require moisture for their growth. Natural substances such as sugar and salt were also used as preservatives because they create high osmotic pressure in foods, making it unpleasant for the survival and reproduction of bacteria. Other examples of natural preservatives include vinegar, syrup, spices, honey and edible oils (Abdulmumeen et al., 2012; Singh et al., 2019).

Chemical compounds are traditionally used to inhibit spoilage in food due to their antimicrobial action against a wide range of microorganisms and their ability to prevent oxidation (Silva and Lidon, 2016). Some chemical preservatives with antimicrobial characteristics include sorbates, benzoates, sulphites, nitrites and nitrates of sodium or potassium while those that act as inhibitors of oxidation are *tert*-butylhydroquinone (TBHQ), propyl gallate (PG), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Silva and Lidon, 2016). Chemical preservatives may be used solely or in combination with other preservatives or preservation methods e.g. pasteurisation (Guedes et al., 2016).

Chemical preservatives are found in most food and food products and their use is regulated by laws which differ from country to country. For example, laws by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) prescribe the type of food they can be used in, maximum amounts permitted in the food and acceptable daily intakes (Table 2.1). However, these regulatory laws are not always complied with as some food producers use preservatives illegally, add concentrations that are higher than the permitted levels or fail to declare the use of preservatives in their

products (del Olmo et al., 2017). Javanmardi et al. (2015) reported that more than half of the soft drinks they tested contained benzoic acid that was higher than the concentration permitted by the European legislation and Iranian Standards Organisation.

Table 2.1. Chemical preservatives found in various foods and beverages

Preservatives	Foods and beverages found	Acceptable daily intake (ADI)
Nitrates and nitrites	Some fresh and frozen vegetables (spinach and lettuce), processed meat and meat products such as ham, bacon, sausages, hot dog, salami, corned beef, etc.	0 - 3.7 mg/kg for nitrates and 0 - 0.07 mg/kg for nitrites (JECFA, 2002)
Sulphur dioxide and sulphites	Grains, dry fruits, vegetables, potatoes and biscuits, beverages such as wine, beer and fruit juice, salted and dried fish, vinegar, brine, glucose syrups, seafood, meat products, etc.	0 - 0.7 mg/kg (JECFA, 2009).
Benzoic acid and benzoates (Sodium, potassium and calcium)	Soft drinks, sauces, fruit juices, non-alcoholic beer, pickled fruits and vegetables, jams and jellies, confectionery, fish and egg based products, condiments and spices, mixed vegetables, etc.	0 - 5 mg/kg (JECFA, 1996).
Sorbic acid and sorbates	Fruit and vegetable juice, cheese, yoghurt, margarine, mayonnaise, sour cream, fresh and dry fruits, baked product such as pies, bread, cakes, icing and toppings, syrups and jellies, smoked and salted fish, salads, dressings, pasta and noodles, etc.	0 - 25 mg/kg (JECFA, 1996).
Antioxidants (BHA, BHT, TBHQ, PG)	Butter, cereals, meat, baked foods, snacks, beer, etc.	BHA: 0 - 0.5 mg/kg, BHT: 0 - 0.3 mg/kg, PG: 0 - 1.4 mg/kg, TBHQ: 0 - 0.7 mg/kg (JECFA, 1996).

Although the application of chemical preservatives in maintaining food quality and safety is important, these chemical preservatives have become contentious in recent years because of the adverse effects associated with their use. Some chemical

preservatives alone are not considered hazardous to humans but their reaction with other molecules can form compounds that are potentially toxic (Bedale et al., 2016). Examples of such preservatives are nitrates and nitrites. Heat (when cooking) reduces nitrates (NO_3) to nitrites (NO_2), nitrites in turn reacts with hydrochloric acid (HCl) in the stomach to form nitrous acid (HNO_2). Nitrous acid reacts with certain amines or amides to form N-nitroso compounds (NOC), most of which are known to be carcinogenic (Silva and Lidon, 2016). Moreover, the reaction between nitrite and haemoglobin (the protein in red blood cells responsible for transporting and distributing oxygen to the body) results in methemoglobinemia; a blood disorder that incapacitates the function of haemoglobin due to high amounts of methemoglobin). Methemoglobinemia often leads to anemia and blue discoloration of the skin, a condition known as cyanosis (Bedale et al., 2016).

Sulphur dioxides and sulphites may cause irritation to the respiratory tract and trigger asthma in susceptible individuals. They may cause allergic reactions such as headache, skin and stomach irritations (Inetianbor et al., 2015). It has also been reported that these preservatives can breakdown thiamine (vitamin B1) leading to thiamine deficiency. This occurs when foods containing sulphites and sulphur dioxide are consumed with foods rich in thiamine. Therefore, they are not used to preserve thiamine containing products (Irwin et al., 2017). The European Food Safety Authority has reported that sorbic acid and sorbates are considered safe and less toxic than other preservatives; however, they are regarded as irritants to the skin, eye and respiratory tract (EFSA, 2014).

Moreover, the mixture of benzoic acid and ascorbic acid are deliberately avoided by beverage companies because they react to produce benzene, a carcinogenic compound (Tzima et al., 2015). Benzoic acid can also irritate the digestive mucous membrane and it has been found that consuming up to 1000 mg/kg, which is higher than acceptable daily intake (Table 2.1) for 5 consecutive days can cause nausea, headache, burning of the oesophagus and reduction of the digestive usage coefficient by inhibiting enzymes such as polypeptidases, pepsin, trypsin and D-

amino acid oxidases (del Olmo et al., 2017). Additionally, the metabolic process can be disrupted during the detoxification of benzoic acid where high amounts of glycine are used. This results in lower levels of glycine which can reduce the production of creatinine, glutamine, urea and uric acid; thereby inducing diarrhoea, tremor, convulsions, hypoactivity, weakness of the muscles, emaciation and metabolic acidosis. Benzoic acid and its salts have also been linked to hyperactivity, especially in children (del Olmo et al., 2017).

2.8. Fruit juice preservation

Chemical preservatives such as sulphur dioxide, benzoic and sorbic acids are widely used to maintain the quality of fruit juice and extend their shelf life. However, they have been reported to cause allergic reactions and other adverse effects in humans (Inetianbor et al., 2015; Guedes et al., 2016). In addition to the use of chemical preservatives, fruit juices are preserved by thermal processing e.g. pasteurisation; a process in which fruit juices undergo heat treatment usually about 90 - 95^{o}C for 15 - 30 s or 77 - 88^{o}C for 25 - 30 s, in order to kill or inactivate microorganisms that may cause spoilage and subsequently illness in humans. This method has been used effectively for many decades to preserve fruit juices. Nonetheless, the nutritional and organoleptic qualities of fruit juices are lost in the process (de Souza et al., 2016).

As a result, consumers demand natural foods, especially those containing bioactive components that are likely to enhance overall health (Hygreeva et al., 2014). Herbs and spices have a wide range of antimicrobial activity and can be used in the control of food spoilage and pathogenic organisms. Therefore, the application of natural antimicrobials from plant extracts and essential oils may be good alternatives for achieving food safety (Calo et al., 2015; Tzima et al., 2015), since products derived from natural sources are innately more tolerated in the human body than chemical preservatives (Silva and Domingues, 2017). This study explored the potential of *Moringa oleifera* and *Xylopia aethiopica* as fruit juice preservatives. These plants were selected from literature based on their rich bioactive properties, especially their antioxidant and antimicrobial properties.

2.9. *Moringa oleifera*

Moringa oleifera Lam. commonly known as horseradish tree, drumstick tree or ben oil tree belongs to a monogeneric family Moringaceae. It is an evergreen shrub of about 5 - 10 m in height (Figure 2.3). Although *M. oleifera* is indigenous to the Himalayan Mountains, Pakistan and India, it is found in the tropical and subtropical regions of the world like Africa, Asia and South America. Moreover, countries like, Mexico, Cambodia, Caribbean Islands and America cultivate it for commercial purposes (Liu et al., 2018).

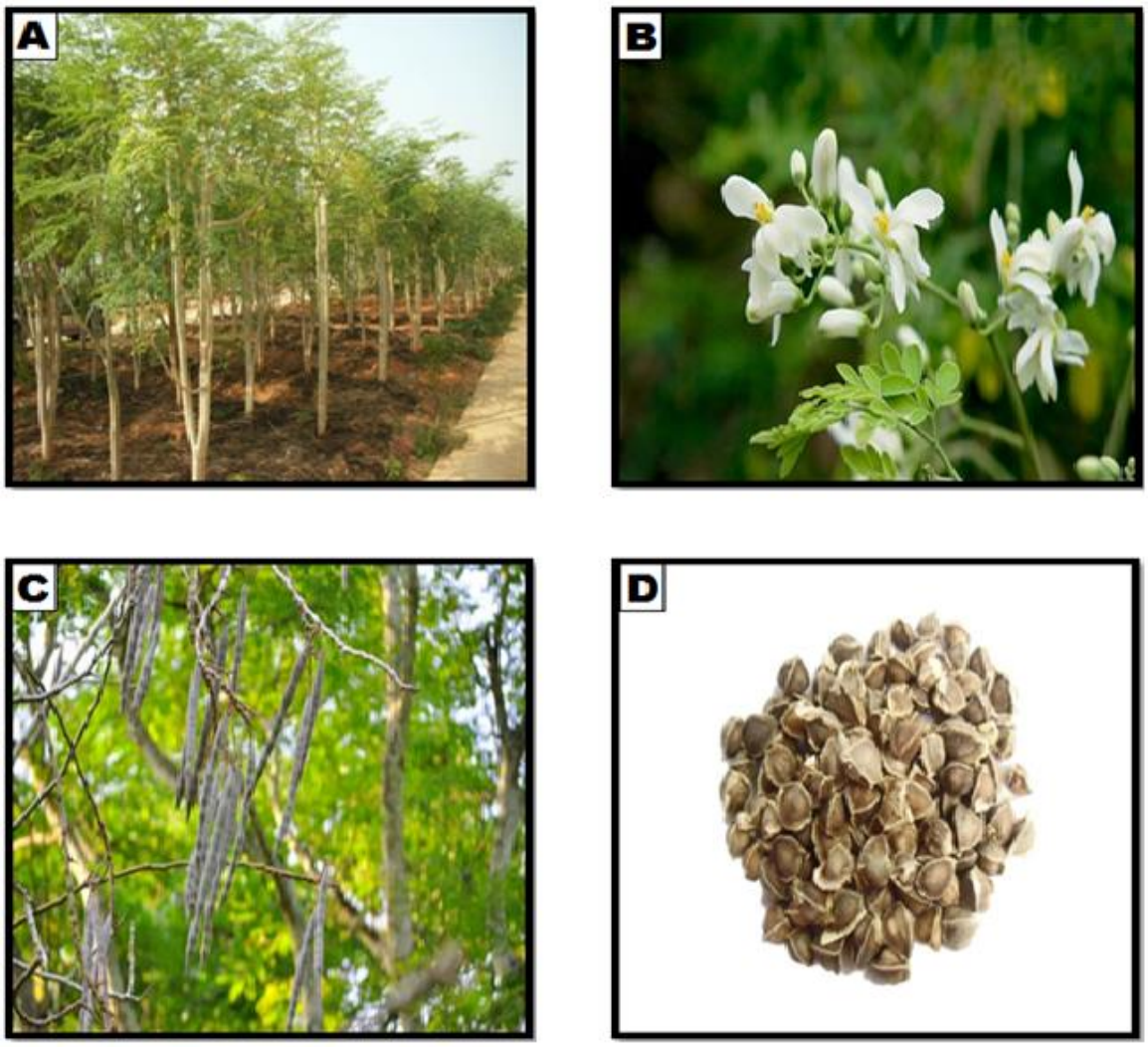

Figure 2.3. *M. oleifera*: (A) whole plant, (B) flowers, (C) mature pods, (D) seeds (Liu et al., 2018).

M. oleifera tree is propagated by seeding or cutting and can be grown at any time of the year. The plant bears fruits and white scented flowers biannually. The leaves are bipinnate or tripinnate compounds with oval shaped leaflets. The fruits, flowers, leaves and pods which contain the seeds are eaten as a vegetable (Alhakmani et al., 2013).

M. oleifera contains a variety of phytochemicals, making it a significant multi-purpose plant. Its application in food, natural medicine, animal fodder, forestry products, fertilizers, living fence, natural coagulants, alley cropping and fuelling, water purification, biopesticide, cosmetics and pharmaceuticals has made it a very popular and extremely valued plant worldwide (Liu et al., 2018; Djande et al., 2018). The leaves of *M. oleifera* are used to treat malnutrition and a wide range of ailments such as bronchitis, ulcers, malaria, fever, symptoms associated with HIV/AIDS (Al_ husnan and Alkahtani, 2016) and has shown potential to treat Type 1 and Type 2 diabetes in rats (Liu et al., 2018). In addition, the extracts of *M. oleifera* have been reported to show antitumor, cardioprotective (Gupta et al., 2018), neuroprotective (Omotoso et al., 2018), anticancer and antimicrobial activities (Al_husnan and Alkahtani, 2016).

M. oleifera is rich in protein, essential amino acids, vitamins, minerals, fatty acids and bioactive compounds. The leaves in particular contain high amounts of calcium, potassium, magnesium, iron, phosphorus, vitamin A, B, C and E, unsaturated fatty acids and essential amino acids such as tyrosine, methionine, threonine, isoleucine, leucine, valine, histidine, phenylalanine, lysine and tryptophan (Falowo et al., 2018; Liu et al., 2018). The aforementioned nutritional contents of *M. oleifera* exceed those of other plants consumed as vegetables or fruits with raw fibre ranging between 7.09 - 35.0 g in per 100 g dried leaves (Falowo et al., 2018; Liu et al., 2018). In food fortification studies, researchers report a major improvement in the quality and nutritional properties of foods such as biscuit (Alam et al., 2014), yoghurts (Kuikman and O'Connor, 2015), cookies (Haneen, 2015) and bread (Bolarinwa et al, 2019) when the powder of *M. oleifera* leaves was used. Researchers have also suggested

the use of *M. oleifera* as a potential natural preservative in foods (Irokanulo et al., 2015; Jayawardana et al., 2015; Gull et al., 2016).

2.10. *Xylopia aethiopica*

Xylopia aethiopica (Dunal) A. Rich. also known as Ethiopian pepper, Negro pepper, African pepper, Guinean pepper, Senegalese pepper and spice tree belongs to the Annonaceae family (Figure 2.4). This evergreen and aromatic tree with a height of about 15 - 30 m and diameter range of 60 - 70 cm originates from the savannah and coastal regions of Africa (Erhirhie and Moke, 2014; Adefegha et al., 2018).

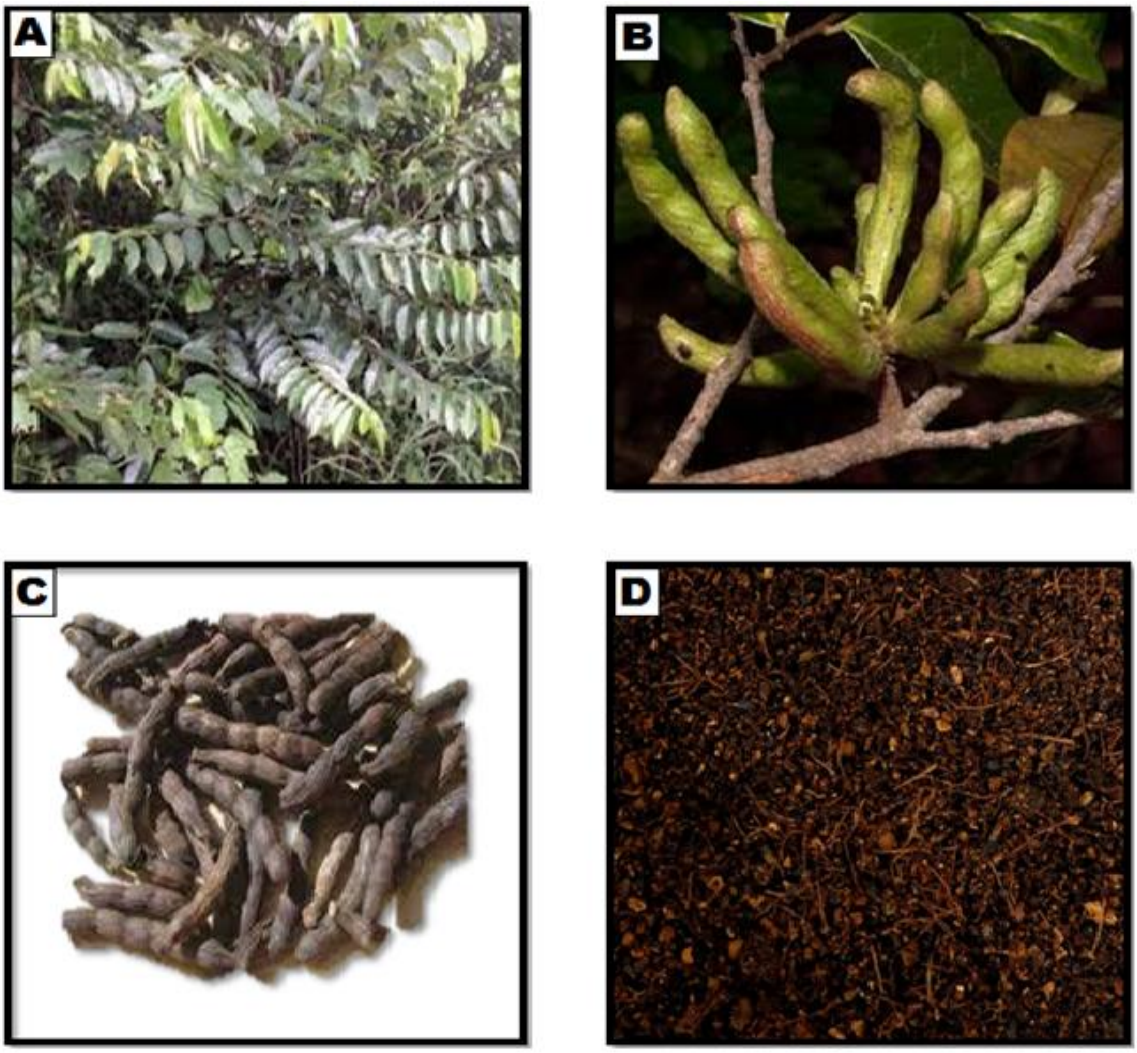

Figure 2.4. *X. aethiopica*: (A) leaves, (B) pods growing in clusters, (C) mature dried fruits, (D) crushed fruits (West African Plants, 2018).

The seeds are encased in the fruits and used in food to replace pepper in some local communities (Adefegha et al., 2018). The fruits which grow in clusters are regarded as the most significant part of the tree due to their multiple uses, especially in medicine and food (Freiesleben et al., 2015).

Different parts of *X. aethiopica* tree (leaves, stem bark, roots, fruits and seeds) are used in traditional medicine to treat various ailments such as dysentery, bronchitis, rheumatism, skin infection, headache, fever, cough and ulceration (Erhirhie and Moke, 2014). In addition to its use for enhancing steroid hormone levels and sperm count (Obembe et al., 2015), studies have demonstrated that the extracts and essential oils of *X. aethiopica* have antihelminthic (Ekeanyanwu and Etienajirhevwe, 2012), antidepressant (Biney et al., 2016), antiinflammatory, (Obiri and Osafo, 2014), antidiabetic (Mohammed et al., 2016), antioxidant (Sokamte et al., 2019) and antimicrobial (Ikeyi et al., 2013) activities. Moreover, the chemical components of *X. aethiopica* include essential oils, resins, annonacin, reberoside, tannins, alkaloids, oxalate and flavonoids (Ekeanyanwu and Etienajirhevwe, 2012).

2.11. Conclusion

The use of chemical preservatives in fruit juices is imperative in order to minimise the health consequences and economic losses that results from oxidation and microbial spoilage. Nonetheless, conventional preservatives applied in fruit juices are known to pose some health hazards to humans. Various studies have indicated that natural plants contain antioxidant and antimicrobial compounds that may be potentially used to extend the quality and shelf life of foods. This necessitates the evaluation of antioxidant and antimicrobial capacity of extracts from *M. oleifera* and *X. aethiopica.*

2.12. References

Abdulmumeen, H.A., Ahmed, N.R. and Agboola, R.S. 2012. Food: Its preservatives, additives and applications. *International Journal of Chemical and Biochemical Science*, 1: 36-47.

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Alam, M.A., Alam, M.J., Hakim, M.A., Huq, A.K.O. and Moktadir, S.M.G. 2014. Development of fiber enriched herbal biscuits: A preliminary study on sensory evaluation and chemical composition. *International Journal of Food Sciences and Nutrition*, 3: 246-250.

Alhakmani, F., Kumar, S. and Khan, S.A. 2013. Estimation of total phenolic content, antioxidant and antiinflammatory activity of flowers of *Moringa oleifera. Asian Pacific Journal of Tropical Biomedicine*, 3(8): 623-627.

Al_husnan, L.A. and Alkahtani, M.D.F. 2016. Impact of moringa aqueous extract on pathogenic bacteria and fungi *in vitro*. *Annals of Agricultural Science,* 61: 247-250.

Al-Kharousi, Z.S., Guizani, N., Al-Sadi, A.M., Al-Bulushi, I.M. and Shaharoona, B. 2016. Hiding in fresh fruits and vegetables: Opportunistic pathogens may cross geographical barriers. *International Journal of Microbiology,* Article ID 4292417: 1-14.

Aneja, K.R., Dhiman, R., Aggarwal, N.K., and Aneja, A. 2014. Emerging preservation techniques for controlling spoilage and pathogenic microorganisms in fruit juices: A review. *International Journal of Microbiology,* 2014: 1-14.

Anvarian, A.H.P., Smith , M.P. and Overton, T.W. 2018. Use of flow cytometry and total viable count to determine the effects of orange juice composition on the physiology of *Escherichia coli. Food Science and Nutrition*, 6(7): 1817-1825.

Bedale, W., Sindelar, J.J. and Milkowski, A. 2016. Dietary nitrate and nitrite: Benefits, risks and evolving perceptions. *Meat Science*, 120: 85-92.

Benner Jr., R.A. 2014. Organisms of concern but not foodborne or confirmed foodborne: spoilage microorganisms. In: *Hazards and diseases*. Motarjemi,

Y., Moy, J. and Todd, E., Eds. Elsevier, Amsterdam, Netherlands: Encyclopaedia of Food Safety, pp. 245-250.

Biney, R.P., Benneh, C.K., Ameyaw E.O., Boakye-Gyasi, E., and Woode, E. 2016. *Xylopia aethiopica* fruit extract exhibits antidepressant-like effect via interaction with serotonergic neurotransmission in mice. *Journal of Ethnopharmacology,* 184: 49-57.

Bolarinwa, I.F., Aruna, T.E. and Raji, A.O. 2019. Nutritive value and acceptability of bread fortified with moringa seed powder. *Journal of the Saudi Society of Agricultural Sciences*, 18: 195-200.

Bukvicki, D., Gottardi, D., Tyagi, A.K., Veljic, M., Marin, P.D., Vujisic, L., Guerzoni, M.E. and Vannini, L. 2014. *Scapania nemorea* liverwort extracts: Investigation on volatile compounds, *in-vitro* antimicrobial activity and control of *Saccharomyces cerevisiae* in fruit juice. *LWT - Food Science and Technology,* 55: 452-458.

Calo, J.R., Crandall, P.G., O'Bryan, C.A. and Ricke**,** S.C. 2015. Essential oils as antimicrobials in food systems: A review. *Food Control,* 54: 111-119.

Chanson-Rolle, A., Braesco, V., Chupin, J. and Bouillot, L. 2016. Nutritional composition of orange juice: A comparative study between French commercial and home-made Juices*. Food and Nutrition Sciences,* 7: 252-261.

Cole, A.T., Akinawoniran, B.O., Olagoke, O.V. and Abubakre, B. 2019. Characterization and isolation of bacteria from selected fresh fruits. *Microbiology Research Journal International*, 26(3): 1-5.

Dal Magro, L., Dalagnol L.M.G., Manfroi, V., Hertz, P.F., Klein, M.P. and Rodrigues, R.C. 2016. Synergistic effects of pectinex ultra clear and lallzyme Beta on yield and bioactive compounds extraction of Concord grape juice. *LWT - Food Science and Technology*, 72: 157-165.

De Souza, E.L., da Cruz Almeida, E.T. and Guedes, J.P.S. 2016. The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: A review. *Comprehensive Reviews in Food Science and Food Safety,* 15: 753.

Del Olmo, A., Calzada, J. and Nuñez, M. 2017. Benzoic acid and its derivatives as naturally occurring compounds in foods and as additives: Uses, exposure and controversy. *Critical Reviews in Food Science and Nutrition*, 57(14): 3084-3103.

Department of Agriculture, Forestry and Fisheries (DAFF). 2012. *Production Guidelines - Grapes*. Republic of South Africa. Available online at https://www.nda. agric.za/docs/Brochures/grapesprod.pdf Accessed on the 14th of May, 2019.

Djande, C.Y.H., Piater, L.A., Steenkamp, P.A., Madala, N.E. and Dubery, I.A. 2018. Differential extraction of phytochemicals from the multipurpose tree, *Moringa oleifera*, using green extraction solvents. *South African Journal of Botany,* 115: 81-89.

Ekeanyanwu, R.C. and Etienajirhevwe, O.F. 2012. *In vitro* anthelmintic potentials of *Xylopia aethiopica* and *Monodora myristica* from Nigeria. *African Journal of Biochemistry Research,* 6: 115-120.

Erhirhie, E.O. and Moke, G.E. 2014. *Xylopia aethiopica*: A review of its ethnomedicinal chemical and pharmacological properties. *American Journal of PharmTech Research*, 4(6): 21-37.

Etebu, E. and Nwauzoma, A.B. 2014. A review on sweet orange (Citrus Sinensis Osbeck): health, diseases and management. *American Journal of Research Communication*, 2: 33-70.

European Food Safety Authority (EFSA). 2014. Scientific opinion on the safety and efficacy of sorbic acid and potassium sorbate when used as technological additives for all animal species: EFSA panel on additives and products or substances used in animal feed (FEEDAP). *EFSA Journal,* 12(7): 3792.

Everhart, S.E. 2010. Upper canopy collection and identification of grapevines (*Vitis*) from selected forests in the south eastern United States. *Papers in Plant Pathology*, 364.

Falowo, A.B., Mukumbo, F.E., Idamokoro, E.M., Lorenzo, J.M., Afolayan, A.J. and Muchenje, V. 2018. Multi-functional application of *Moringa oleifera* Lam. in

nutrition and animal food products: A review. *Food Research International,* 106: 317-334.

Fatta Del Bosco, S., Abbate, L., Mercati, F., Napoli, E. and Ruberto, G. 2020. Essential oils in Citrus. In: *The Citrus Genome*: *Compendium of Plant Genomes*. Gentile, A., La Malfa, S., Deng, Z., Eds. Italy: Springer Cham, pp. 221-223.

Favela-Hernández, J.M.J., González-Santiago, O., Ramírez-Cabrera, M.A., Esquivel-Ferriño, P.C. and Camacho-Corona, M.R. 2016. Chemistry and pharmacology of *Citrus sinensis*: A review*. Molecules,* 21(2): 247.

Florida Department of Citrus, 2012. Available online at https://www.yelp.com/biz/florida-department-of-citrus-bartow-2. Accessed on the 8th of April, 2019.

Food and Agriculture Organisation (FAO) of the United Nations. 2012. Save Food: global initiative on food loss and waste reduction. Available online at http://www.fao.org/save-food/resources/keyfindings/infographics/roots/en/ Accessed on the 6th of April, 2019.

Food and Agricultural Organisation (FAO) **of the United Nations**. 2017. Global production of crops: Available online at http://www.fao.org/faostat/en/#data/QC Accessed on the 6th of April, 2019.

Food Safety Authority of Ireland (FSAI). 2018. PepsiCo recalls a batch of Tropicana Trop50 Multivitamins Juice due to fermentation. Available online at https:// www.fsai.ie/news_centre/food_alerts/tropicana.html Accessed on the 24th of January, 2020.

Freiesleben, S.H., Soelberg, J. and Jäger, A.K. 2015. Medicinal plants used as excipients in the history in Ghanaian herbal medicine. *Journal of Ethnopharmacology*, 174: 561-568.

Ghan, R., Petereit, J., Tillett, R.L., Schlauch, K.A., Toubiana, D., Fait, A. and Cramer, G.R. 2017. The common transcriptional subnetworks of the grape berry skin in the late stages of ripening. *BMC Plant Biology*, 17: 94.

Gomes, T.M., Toaldo, I.M., Haas, I.C.S., Burin, V.M., Caliari, V., Luna, A.S., de Gois, J. S. and Bordignon-Luiz, M.T. 2019. Differential contribution of grape peel, pulp, and seed to bioaccessibility of micronutrients and major

polyphenolic compounds of red and white grapes through simulated human digestion. *Journal of Functional Foods,* 52: 699-708.

Goulas, V. and Manganaris, G.A. 2012. Exploring the phytochemical content and the antioxidant potential of Citrus fruits grown in Cyprus. *Food Chemistry,* 131: 39-47.

Guedes, J.P.S., da Costa Medeiros, J.A., de Souza e Silva, R.S., de Sousa, J.M.B., da Conceição, M.L. and de Souza, E.L. 2016. The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices. *International Journal of Food Microbiology,* 238: 183-192.

Gull, I., Javed, A., Aslam, M.S., Mushtaq, R. and Athar, M.A. 2016. Use of *Moringa oleifera* flower pod extract as natural preservative and development of SCAR marker for its DNA based identification. *BioMed Research International,* 2016: 1-12.

Gupta, S., Jain, R., Kachhwaha, S. and Kothari, S.L. 2018. Nutritional and medicinal applications of *Moringa oleifera* Lam.—Review of current status and future possibilities. *Journal of Herbal Medicine*, 11: 1-11.

Haneen, H.S.M. 2015. Effect of dried *Moringa oleifera* leaves on the nutritional and organoleptic characteristics of cookies. *Alexandria Science Exchange Journal,* 36(4): 297-305.

Hernández, A., Pérez-Nevado, F., Ruiz-Moyano, S., Serradilla, M.J., Villalobos, M.C., Martín, A. and Córdoba, M.G. 2018. Spoilage yeasts: What are the sources of contamination of foods and beverages? *International Journal of Food Microbiology,* 286: 98-110.

Hygreeva, D., Pandey, M.C. and Radhakrishna, K. 2014. Potential applications of plant based derivatives as fat replacers, antioxidants and antimicrobials in fresh and processed meat products: A review. *Meat Science,* 98: 47-57.

Hyson, D.A. 2016. The review and critical analysis of the scientific literature related to 100% fruit juice and human health. *Advance Nutrition,* 6: 37-51.

Ikeyi, A.P., Ogbonna, A.O., Ibekwe, R.O. and Ugwu, O.P.C. 2013. Antimicrobial activity of *Xylopia aethiopica* (UDA) on *Escherichia coli* and *Staphylococcus*

aureus isolates from gastro enteritic patients. *International Journal of Life Science Biotechnology Pharma Research*, 2: 330-338.

Inetianbor, J.E., Ykubu, J.M. and Ezeonu, S.C. 2015. Effects of food additives and preservatives on man: A review. *Asian Journal of Science and Technology*, 6(2): 1118-1135.

International Federation of Fruit Juice Producers (**IFU)**. 2017. Fruit Juice - Nutrition and Health: An IFU scientific review. Available online at https://cdn.ymaws.com/ifu-fruitjuice.com/resource/resmgr/stc/fruit_juice_nutrition__healt.pdf Accessed on the 14th of May, 2019.

Irokanulo, E.O., Egbezien, I.L. and Orwa, S.O. 2015. Use of *Moringa oleifera* in the preservation of fresh tomatoes. *Journal of Agriculture and Veterinary Science,* 8(2): 127-132.

Irwin, S.V., Fisher, P., Graham, E., Malek, A. and Robidoux, A. 2017. Sulfites inhibit the growth of four species of beneficial gut bacteria at concentrations regarded as safe for food. *PLoS ONE,* 12(10): e0186629.

Javanmardi, F., Nemati, M., Ansarin, M. and Arefhosseini, S.R. 2015. Benzoic and sorbic acid in soft drink, milk, ketchup sauce and bread by dispersive liquid-liquid microextraction coupled with HPLC. *Food Additives and Contaminants: Part B,* 8(1): 32-39.

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S. and Weththasinghe, P. 2015. Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. *LWT - Food Science and Technology*, 64: 1204-1208.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 1996. Evaluation of certain food additives and contaminants: forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives. *WHO Technical Report Series*, 868.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2002. Evaluation of certain food additives and contaminants: fifty-ninth report of the Joint FAO/WHO Experts Committee on Food Additives. *WHO Technical Report Series,* 913. 20-32.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2009. Evaluation of certain food additives and contaminants: sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. *WHO Technical Report Series*, 891.

Kuikman, M. and O'Connor, C.P. 2015. Sensory evaluation of moringa-probiotic yogurt containing banana, sweet potato or avocado. *Journal of Food Research*, 4: 165-171.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

Maciel, N.O.P., Piló, F.B., Freitas, L.F.D., Gomes, F.C.O., Johann, S., Nardi, R.M.D., Lachance, M.A. and Rosa, C.A. 2013. The diversity and antifungal susceptibility of the yeasts isolated from coconut water and reconstituted fruit juices in Brazil. *International Journal of Food Microbiology,* 160: 201-205.

Mahgoub, S.A.M. and El-Shourbagy, G.A. 2015. Microbiological and physicochemical criteria of fruit juices sold in Egypt: Incidence of spore-forming bacteria. *Emirates Journal of Food and Agriculture*, 27(11): 864-871.

Mannucci, C., Calapai, F., Cardia, L., Inferrera, G., D'Arena, G., Di Pietro, M., Navarra, M., Gangemi, S., Spagnolo, E.V. and Calapai, G. 2018. Clinical pharmacology of *Citrus aurantium* and *Citrus sinensis* for the treatment of anxiety. *Evidence-based Complementary and Alternative Medicine*, 2018(7): 1-18.

Martínez-Gonzáles, N.E. and Castillo, A. 2016. Chapter 11 – Safety of fresh-squeezed juices. In: *Food Hygiene and Toxicology in Ready-to-Eat Foods*. Kotzekidou, P., Ed. USA: Academic Press. pp. 183-208.

Mohammed, A., Koorbanally, N.A. and Islam, Md.S. 2016. Antidiabetic effect of *Xylopia aethiopica* (Dunal) A. Rich. (Annonaceae) fruit acetone fraction in a type 2 diabetes model of rats. *Journal of Ethnopharmacology*, 180: 131-139.

Nayik, G.A., Amin, T. and Bhat, S. 2013. Microbial analysis of some fruit juices available in the markets of Kashmir valley, India. *Asian Journal of Microbiology, Biotechology and Environmental Sciences*, 15(4): 733-737.

Obembe, A.O., Ofutet, E.O., Okpo-ene, A.I., Okon, V.E. and Eyong, E.E. 2015. The effect of aqueous extract of *Xylopia aethiopica* (nigro pepper) on some haematological parameters in albino rats. *World Journal of Pharmaceutical Research,* 4(10): 2576-2583.

Obiri, D.D., Osafo, N., Ayande, P.G. and Antwi, A.O. 2014. *Xylopia aethiopica* (Annonaceae) fruit extract suppresses Freund's adjuvant-induced arthritis in Sprague-Dawley rats. *Journal of Ethnopharmacology*, 152: 522-531.

Oluwole, O.A., David, O.M., Falegan, C.R., Awojuyigbe, B. and Olajide, O.M. 2016. Microbiological and physicochemical properties of commercial seal tampered refrigerated fruit juices. *International Journal of Biological Research,* 4(1): 21-24.

Omotoso, G.O., Gbadamosi, I.T., Olajide, O.J., Dada-Habeeb, S.O., Arogundade, T.T. and Yawson, E.O. 2018. *Moringa oleifera* phytochemicals protect the brain against experimental nicotine-induced neurobehavioral disturbances and cerebellar degeneration. *Pathophysiology,* 25: 57-62.

Park, Y.S., Kim, S.K., Kim, S.Y., Kim, K.M. and Ryu, C.M. 2019. The transcriptome analysis of the *Arabidopsis thaliana* in response to the *Vibrio vulnificus* by RNA-sequencing. *PLoS ONE,* 14(12): e0225976.

Pascual-Ahuir, M.N., Lerma-García, M.J., Simó-Alfonso, E.F. and Herrero-Martínez, J.M. 2015. Quality control of fruit juices by using organic acids determined by capillary zone electrophoresis with poly (vinyl alcohol)-coated bubble cell capillaries. *Food Chemistry,* 188: 596-603.

Petruzzi, L., Corbo, M.R., Sinigaglia, M. and Bevilacqua, A. 2017. Microbial spoilage of foods: Fundamentals. In: *The Microbiological Quality of Food: Foodborne Spoilers.* Bevilacqua, A., Corbo, M.R. and Sinigaglia, M., Eds. València, Spain: Woodhead Publishing Series in Food Science, Technology and Nutrition, pp. 1-21.

Rawat, S. 2015. Food Spoilage: Microorganisms and their prevention. *Asian Journal of Plant Science and Research*, 5(4): 47-56.

Silva, F. and Domingues, F.C. 2017. Antimicrobial activity of coriander oil and its effectiveness as food preservative. *Critical Reviews in Food Science and Nutrition*, 57(1): 35-47.

Silva, M.M. and Lidon, F.C. 2016. Food preservatives - An overview on applications and side effects. *Emirates Journal of Food and Agriculture,* 28(6): 366-373.

Singh, P.K., Singh, R.P., Singh, P. and Singh, R.L. 2019. Food hazards: Physical, chemical and biological. In: *Food Safety and Human Health.* Singh, R.L. and Mondal, S., Eds. India: Academic Press, pp. 15-65.

Snyder, A.B. and Worobo, R.W. 2018. The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers. *Food Control,* 85: 144-150.

Sokamte, T.A., Mbougueng, P.D., Tatsadjieu, N.L. and Sachindra, N.M. 2019. Phenolic compounds characterization and antioxidant activities of selected spices from Cameroon. *South African Journal of Botany,* 121: 7-15.

Tzima., K., Makris, D., Nikiforidis, C.V. and Mourtzinos I. 2015. Potential use of rosemary, propolis and thyme as natural food preservatives. *Journal of Nutrition and Health,* 1(1): 6.

U.S. Food and Drug Administration (USFDA). 2001. Hazard analysis and critical control point (HACCP): procedures for the safe and sanitary processing and importing of juices; final rule. *Federal Register*, 66: 6138-6202.

Wang, H., Hu, Z., Long, F., Guo, C., Niu, C., Yuan, Y. and Yue, T. 2016. Combined effect of sugar content and pH on the growth of a wild strain of *Zygosaccharomyces rouxii* and time for spoilage in concentrated apple juice. *Food Control,* 59: 298-305.

West African Plant, 2018. A Photo Guide. Available online at http://www.westafricanplants.senckenberg.de/root/index.php?pageid=5. Accessed on the 24th of April, 2019.

Chapter Three

Phytochemical screening, antioxidant activity and total phenolic content of *Moringa oleifera* and *Xylopia aethiopica* extracts

3.1. Abstract

The significance of phenolic compounds in the antioxidant activity of herbs and spices has increasingly been recognised over the years, not just for their potential to prevent the risks of chronic and degenerative diseases induced by oxidative stress, but also for their importance as food additives. Consequently, bioactive compounds with good antioxidant activity in herbs and spices can be used in fruit juices to improve quality and prolong shelf life. This study analysed the phytochemical contents of 50% methanol, 50% acetone and water extracts of *Moringa oleifera* leaves and *Xylopia aethiopica* fruits. The antioxidant activity and total phenolic content of the plant extracts was also determined using the DPPH and Folin Ciocalteu methods respectively. Qualitative phytochemical analyses indicated the presence of flavonoids, terpenoids, tannins, glycosides, alkaloids and saponins in all the tested extracts except the water extract of *M. oleifera* and all the extracts of *X. aethiopica* which lacked alkaloids and terpenoids respectively. The best antioxidant activity and total phenolic content of *M. oleifera* was shown by 50% methanol extract with an IC_{50} of 48.09 µg/mL and GAE/g of 110.0 mg while 50% acetone extract had the highest activity for *X. aethiopica* (IC_{50} = 19.10 µg/mL and GAE/g = 173.2 mg). The extract with the strongest antioxidant activity (IC_{50}) also had the highest total phenolic content (GAE/g). The water extracts of both plant species had the least antioxidant activity and total phenolic content. The findings in this study demonstrate that plants like *M. oleifera* and *X. aethiopica* are rich in phenolic compounds with good antioxidant activity. Further investigation on the use of these plants as natural antioxidants in fruit juice is recommended.

Keywords: Oxidation, spoilage, phytochemicals, natural antioxidants, spices, fruit juice preservative.

3.2. Introduction

Oxidation is a normal process that takes place in biological systems. It is usually accompanied by the formation of toxic compounds that lead to oxidative stress as a result of the accumulation of free radicals when their production surpasses their removal from cells and tissues (Saeed et al., 2012; Peña-Bautista et al., 2019). In humans, the resultant oxidative stress has been linked to the development of several diseases like cancer, heart diseases, cataracts, Parkinson's and Alzheimer's disease, gastric problems and debilitation of the body's immune system due to aging (Guldiken et al., 2018).

Similarly, the occurrence of oxidation during food processing and storage causes significantly unpleasant alterations in the sensory and nutritional qualities of foods rendering them detrimental for human consumption and also shortening their shelf life (Souza et al., 2018). Although all kinds of food and food products undergo oxidation when exposed to air, fatty foods, especially those containing unsaturated fats (e.g. oils and margarines), fruits and their derivatives are the most susceptible to oxidation (Silva and Lidon, 2016). Despite the diverse antioxidant compounds e.g. phenolic compounds, vitamin C and E present in fruit juices, they may still exhibit the effects of oxidation because their antioxidant capacity may not be sustainable during storage (Ephrem et al., 2018).

Antioxidants are compounds that inhibit the oxidation process in other oxidizable compounds (Ephrem, 2018). Their application in food preservation is of paramount importance. The most common antioxidants used in food preservation are chemical compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ). However, owing to their carcinogenic and toxicology effects, restricted usage and consumers' demand for natural products, there has been a ceaseless quest for natural antioxidants, especially from natural plant sources (Tzima et al., 2015; Khatua et al., 2017c).

The major classes of plant secondary metabolites found generously in plants are phenols, a group of chemical compounds with one or more hydroxyl groups attached to aromatic rings (Figure 3.1). The hydroxyl groups of phenolic compounds is a good hydrogen donor and can function as antioxidants by reacting with free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) in a chain reaction. These reactions interrupt the cycle that leads to the formation of new free radicals or chelating metal ions such as iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{2+}) that act as catalysts in oxidation (Vladimir-Knežević et al., 2011; Yao et al., 2016).

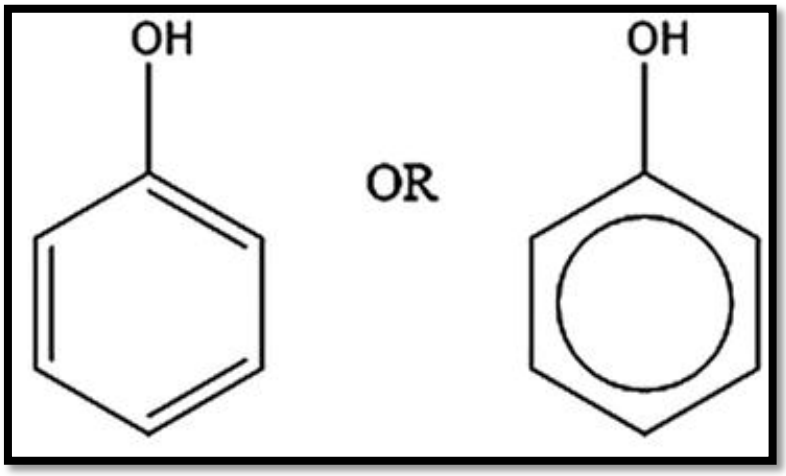

Figure 3.1. The basic structure of phenolic compounds

In addition to acting as antioxidants, phenolic compounds display many biological characteristics such as antiinflammatory, antiallergy, antiaging, antimicrobial, antiviral, etc. and help to reduce the risks of chronic and degenerative diseases. Phenolic compounds e.g. flavonoids, phenolic acids and terpenes are abundantly present in herbs and spices, accounting for their antioxidant activity (Yao et al., 2016; Guldiken et al., 2018).

Various studies have reported the phenolic and antioxidant properties of herbs and spices (Harsha et al., 2013; Vallverdú-Queralt et al., 2014, Sepahpour et al., 2018; Sokamte et al., 2019). Herbs and spices are richly packed with phytochemicals that are not necessarily essential in human nutrition but provide significant benefits that

promote good health like immunoregulatory (Khazdair et al., 2018), anticancer, antiinflammatory, antidiabetic, antihypertensive, antioxidant, etc. (Bower et al., 2016; Guldiken et al., 2018). Besides their use in averting and treating all manners of diseases they have been used for centuries to enhance the aroma, taste and colour as well as extend the shelf life of foods (Bode and Dong, 2015).

Moringa oleifera leaves are consumed as food and medicine because of their rich nutritional and phytochemical contents (Liu et al., 2018). *M. oleifera* leaves have been used to fortify various foods including yoghurt (Kuikman and O'Connor, 2015) and cookies (Haneen, 2015). Moreover, Jayawardana et al. (2015) has shown that lipid oxidation can be impeded and microbial growth reduced in chicken sausages incorporated with *M. oleifera* leaves at 0.50% concentration. The researchers suggest that *M. oleifera* leaves can be potentially used to prolong the shelf life of chicken sausages during cold storage without affecting the sensory qualities.

Xylopia aethiopica fruit is a spice used to improve the sensory characteristics of foods. It is also used to prevent and cure different diseases in traditional medicine (Ekeanyanwu and Etienajirhevwe, 2012; Adefegha et al., 2018). *X. aethiopica* is abundant in antioxidants (Sokamte et al., 2019) and its inhibitory activity on the growth of some food spoilage and pathogenic organisms such as *Saccharomyces cerevisiae, Penicillium funiculosum, Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus* have been reported, suggesting its use as a natural preservative in food (Ogbonna et al., 2013).

Therefore, the purpose of this study was to determine the phytochemical constituents, antioxidant activity and total phenolic content of *M. oleifera* and *X. aethiopica* extracts and indicate their potential as natural alternatives in the preservation of fruit juices.

3.3. Methods

3.3.1. Preparation and optimization of plant extracts

Dry *M. oleifera* leaf powder was commercially sourced from Limpopo while *X. aethiopica* fruits were purchased from a spice company (Spice club) in Mpumalanga, South Africa. The fruits of *X. aethiopica* were air dried till a constant weight was reached, ground into coarse powder using a grinder and stored in clean airtight glass bottles at room temperature until used. The powdered samples (*M. oleifera* and *X. aethiopica*) were initially extracted with absolute acetone or methanol but the obtained extract was very oily and could not be re-dissolved in Dimethyl sulfoxide (DMSO), methanol or acetone at 2, 5, 10 and 50%. Nevertheless, the extracts were successfully re-dissolved in their solvent of extraction (100% methanol or acetone) and this made it impossible to carry on with further assays, especially the antimicrobial activity test because the test organisms may show susceptibility to absolute solvents (Chander et al., 2015) and any positive result obtained from the study cannot be validated.

In order to remove the lipids, the plant materials were defatted by soaking in hexane overnight with constant shaking. The hexane was removed afterwards and plant materials were left to air dry before extracting separately with absolute methanol and acetone. However, attempt to re-constitute the resultant extracts in 2, 5, 10 and 50% DMSO, methanol or acetone was still unsuccessful. Next, the plant materials were macerated in hexane and placed on a shaker. After 2 hours, the solvent was removed and replaced with another fresh solvent. This process was repeated three times followed by extraction with pure methanol and acetone but the result remained the same.

Consequently, solvent mixtures acetone and water (1:1) or methanol and water (1:1) were used for plant extraction. This was done in order to dilute the concentration of organic solvents so that the amount of oily or non-polar compounds extracted would be reduced, since water allows a more efficient extraction of polar compounds (Braham et al., 2019).

Fifty grams of *M. oleifera* and *X. aethiopica* were weighed and transferred into sterile bottles where 250 mL of 50% acetone, 50% methanol and distilled water were separately added and kept at room temperature with continuous shaking. The solvents were filtered after 12 hours of maceration using Whatman No. 1 filter paper and soaked again with fresh solvents. This process was repeated until the solvents added to the plant material became colourless. The extracts were concentrated and the solvents removed using a Genevac Rocket evaporator system (Figure 3.2) while the aqueous extracts were lyophilized. The plant extracts were weighed and stored in the refrigerator at 4^{o}C until required for further analysis.

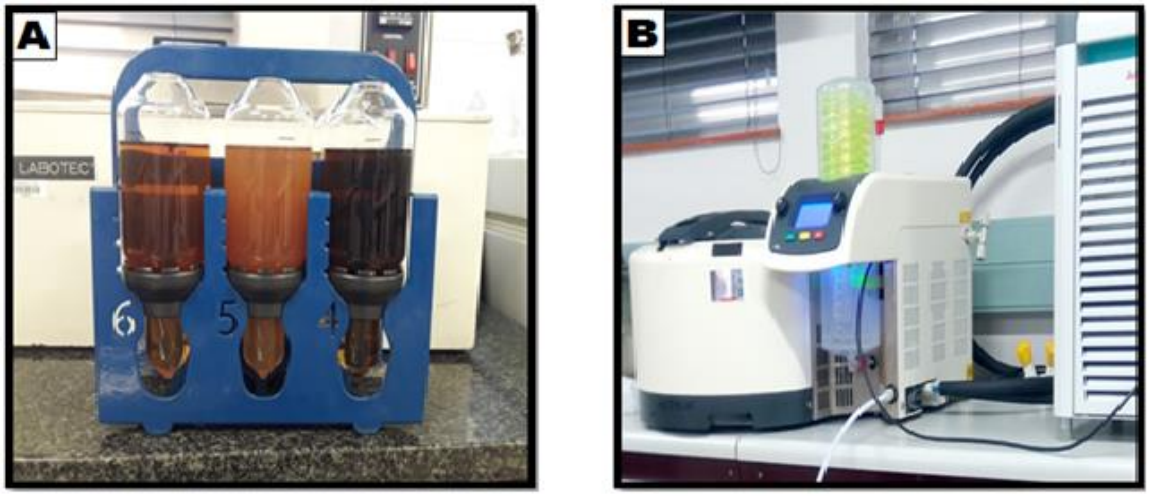

Figure 3.2. (A) *Xylopia aethiopica* extracts in flasks ready to be concentrated (B) Genevac rocket evaporator

The percentage yields of extraction were calculated using the following equation:

$$\text{Extract yields (\%)} = \frac{\text{mass of extract obtained (g)}}{\text{mass of plant sample used (g)}} \text{ x } 100$$

3.3.2. Phytochemical screening of *M. oleifera* and *X. aethiopica* extracts

Qualitative phytochemical analyses described by Al Ghasham et al. (2017) were used to screen for the presence of alkaloids, tannins, saponins, glycosides, flavonoids and terpenoids in the plant extracts.

3.3.2.1. Detection of alkaloids

Two millilitres of 1% hydrochloric acid were added to 50 mg of plant extract and heated in a water bath until steaming. Thereafter, 1 mL of the acidified extract was taken into a test tube and six drops of Wagner's reagent were added. The solution was observed for a brownish red precipitate.

3.3.2.2. Detection of tannins

A few drops of 1% lead acetate were added into 2 mL of plant extract (50 mg/mL). A greenish grey colour indicated a positive result.

3.3.2.3. Detection of saponins

One millilitre of plant extract (50 mg/mL) was added to 5 mL distilled water and shaken vigorously. The formation of insistent froth indicated the presence of saponins.

3.3.2.4. Detection of glycosides

Fifty milligrams of plant extract was dissolved in 2 mL of chloroform. Subsequently, a few drops of sulphuric acid (H_2SO_4) solution were carefully added by the side of the test tubes. The presence of glycosides was indicated by a brown ring formation at interphase.

3.3.2.5. Detection of flavonoids

A millilitre of plant extract (50 mg/mL) was mixed with 1 mL of 10% lead acetate solution and observed for a yellow colour precipitate.

3.3.2.6. Detection of terpenoids

Chloroform (1 mL) was mixed with 2.5 mL of plant extract (50 mg/mL) and then 1.5 mL of concentrated sulphuric acid (H_2SO_4) was added. The formation of a reddish brown colour at interface showed a positive result.

3.3.3. Determination of antioxidant activity of *M. oleifera* and *X. aethiopica* extracts

The antioxidant activity of each extract was determined by the free radical-scavenging method using a stable radical 2, 2- dipheny- 1- picrylhydrazyl (DPPH) assay described by Khatua et al. (2017c) with some modifications. A standard solution of ascorbic acid was prepared by adding 5 mL of methanol into 5 mg of ascorbic acid, previously weighed in a clean McCartney bottle and wrapped in aluminium foil. The mixture was vortexed for complete dissolution to make a 1 mg/mL concentration. To prepare a 1mM stock solution of 2, 2- dipheny- 1- picrylhydrazyl (DPPH) reagent, 50 mL of methanol was added to 20mg of DPPH. The 0.1 mM DPPH working solution was made by transferring 20 mL of the stock solution into a 200 mL volumetric flask, then the solution was diluted to volume with methanol.

All tests were carried out in a 96-well (transparent flat bottom) micro-titre plate and each test was performed in triplicate. A multi-channel pipette was used to transfer 100 µL of methanol into all the wells in row 1 to 8 (Figure 3.3).

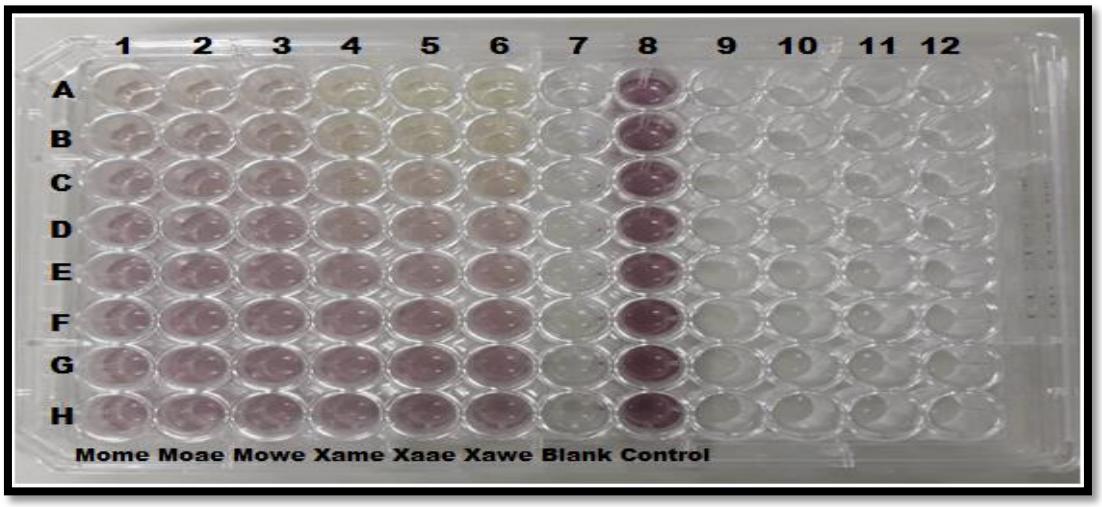

Figure 3.3. Antioxidant activity of **Mome:** *M. oleifera* methanol extract, **Moae:** *M. oleifera* acetone extract, **Mowe:** *M. oleifera* water extract, **Xame:** *X. aethiopica* methanol extract, **Xaae:** *X. aethiopica* acetone extract, **Xawe:** *X. aethiopica* water extract at different concentrations (top to bottom)

An additional 100 µL of methanol was added into the wells in column 7 which served as the blank. Wells in column 8 served as the control. A two-fold dilution was carried out by pipetting 100 µL from the test samples (250 ug/mL) and transferred into the wells in the first row 1 to 6 to give an initial concentration of 125 ug/mL. The starting concentration for ascorbic acid was 15 ug/mL. Afterwards, 100 µL was pipetted from the first column 1 to 6 and transferred into the next corresponding columns. The solution was mixed with a pipette about 4-5 times and 100 µL was again transferred to the third column. The process was repeated until the last column from which 100 µL was discarded. One hundred microliter of DPPH working solution (0.1 Mm) was added into all the wells containing the test samples, ascorbic acid (standard solution) and control to give a total volume of 200 µL in each well. The micro-titre plate was covered with a lid, wrapped in aluminium foil and incubated in a dark at room temperature for 30 minutes. Absorbance was measured at wavelength of 517 nm with a spectrophotometer (Spectramax *M2*^{e}) and the percentage of radical scavenging activity was obtained using the following equation:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

The percentage scavenging activity was plotted against the concentration of the samples and the IC_{50}, i.e. the concentration of sample at which 50% of the DPPH radical was scavenged, were determined by linear regression using the GraphPad Prism software version 5.0.

3.3.4. Determination of total phenolic content of *M. oleifera* and *X. aethiopica* extracts

The total phenolic content of *M. oleifera* and *X. aethiopica* was determined using the Folin- Ciocalteu method described by Waterhouse, (2003) with slight modification. The stock solution of gallic acid standard was made by mixing 500 mg of gallic acid powder with 10 mL of ethanol in a 100 mL volumetric flask, then the solution was made up to volume with distilled water. Subsequently, six volumetric flasks (100 mL) were labelled (0, 1, 2, 3, 5 and 10) and 1, 2, 3, 5 and 10 mL was pipetted from the

gallic acid stock solution and added to the matching flasks. Afterwards the flasks were filled to volume with distilled water thereby making the phenol concentrations in the flasks 0, 50, 100, 150, 250 and 500 mg/L gallic acid respectively. The flask labelled '0' was left untouched and served as a blank. Sodium carbonate solution was prepared by dissolving 50 g of anhydrous sodium carbonate in 250 mL of distilled water and slightly warmed for complete dissolution.

To separate test tubes, 1.58 mL of distilled water, 20 µL from each flask containing different concentrations of gallic acid and 100 µL of 2N Folin-Ciocalteu reagent (Sigma) were added. The solution was mixed using a vortex and left for 8 minutes to react. Thereafter, 300 µL of sodium carbonate solution ($NaCO_3$) was added, mixed and incubated for 2 hours at 20^{o}C. The absorbance of each solution was measured using a spectrophotometer at a wavelength of 765 nm. The same procedure was replicated for the plant extracts at 1 mg/mL concentration and each test was carried out in triplicate. The total phenol values were expressed as milligram Gallic Acid Equivalent per gram (GAE/g) dry extract weight and the absorbance mean was plotted against the concentration.

3.4. Results and discussion

3.4.1. Extract yields from *M. oleifera* and *X. aethiopica*

Table 3.1 shows the extract yields of *M. oleifera* and *X. aethiopica.* The methanol and acetone extract of *M. oleifera* respectively yielded a brown and green jelly-like mass while the water extract yielded a brown dry mass. The acetone extract of *M. oleifera* had the highest yield followed by the methanol extract then the water extract. The polarity of solvents may have contributed to the variation in colour and masses. Acetone being the most non-polar solvent used probably allowed the extraction of other non-polar compounds like chlorophyll, fats and oils thus affecting the colour of the acetone extract of *M. oleifera* and increasing its yield. Conversely, water having the highest polarity index may have extracted only polar compounds thereby resulting in low yield (Ngo et al., 2017).

Table 3.1. Extract yields of *M. oleifera* and *X. aethiopica*

	M. oleifera			***X. aethiopica***		
	Methanol extract	**Acetone extract**	**Water extract**	**Methanol extract**	**Acetone extract**	**Water extract**
Mass of plant samples used (g)	50	50	50	50	50	50
Mass of extracts obtained (g)	29.96	33.97	9.65	23.13	26.02	5.40
Yield of extracts (%)	59.92	67.94	19.30	46.26	52.04	10.80
Colour and texture of extracts	Brown jelly-like mass	Green jelly-like mass	Brown dry mass	Brown dry mass	Brown dry mass	Brown dry mass

All the extracts of *X. aethiopica* yielded a brown dry mass with the acetone extract having the highest yield (Table 3.1). The difference in the masses of *X. aethiopica* extracts confirm that the yields depend greatly upon the solvents used for extraction (Ngo et al., 2017) and in this case 50% acetone was a better solvent for extraction from *M. oleifera* and *X. aethiopica* than 50% methanol or water alone.

3.4.2. Qualitative phytochemical contents of *M. oleifera* and *X. aethiopica* extracts

The phytochemical constituents of *M. oleifera* and *X. aethiopica* extracts are presented in Table 3.2 which also shows screening results from other studies. The results indicate that alkaloids, tannins, saponins, glycosides, flavonoids and terpenoids were present in all the extracts of *M. oleifera,* except the water extract which was devoid of alkaloids. The contents of the water and methanol extracts of *M. oleifera* leaves were consistent with the findings of Vinoth et al. (2012) and Shanmugavel et al. (2018). However, the number and type of phytochemical groups present differed from those in other studies (Table 3.2).

Table 3.2. Phytochemical contents of *M. oleifera* and *X. aethiopica* extracts

	Alk	Tan	Sap	Gly	Fla	Ter
***M. oleifera* methanol extract**	+	+	+	+	+	+
Shanmugavel et al. (2018)	+	+	+	+	+	+
Maqsood et al. (2017)	+	+	+	-	+	-
***M. oleifera* acetone extract**	+	+	+	+	+	+
Padmalochana (2018)	-	+	-	+	+	+
***M. oleifera* water extract**	-	+	+	+	+	+
Vinoth et al. (2012)	-	+	+	+	+	+
Maqsood et al. (2017)	+	+	-	-	-	-
Patel et al. (2014)	+	-	+	-	+	-
***X. aethiopica* methanol extract**	+	+	+	+	+	-
Ekeanyanwu and Etienajirhevwe, (2012)	+	+	+	+	+	-
***X. aethiopica* acetone extract**	+	+	+	+	+	-
Ngwoke et al. (2015)	+	+	-	+	+	+
***X. aethiopica* water extract**	+	+	+	+	+	-
Ekeanyanwu and Etienajirhevwe, (2012)	+	+	+	+	+	-
Ngwoke et al. (2015)	+	+	+	+	+	-
Ekeh et al. (2015)	+	-	+	+	+	-

Alk: Alkaloids, **Tan:** Tannins, **Sap:** Saponins, **Gly:** Glycosides, **Fla:** Flavonoids, **Ter:** Terpenoids, **+:** Positive test, **-:** Negative test.

All *X. aethiopica* extracts contained the investigated compounds, except terpenoids. This supports the findings of Ekeanyanwu and Etienajirhevwe, (2012) who reported the presence of flavonoids, alkaloids, tannins, glycosides and saponins in the water and methanol extract of *X. aethiopica*. Environmental factors from the source of plant material contribute to the nature and quantities of secondary metabolites present in plants (Kumar et al., 2017). This may explain the contradictions between the findings of this study and other studies (Table 3.2).

Plants are known to produce diverse secondary metabolites that function as their defence systems against biotic and abiotic stresses (Yang et al., 2018). This means that the level of stress a particular plant is exposed to, determines the type and concentration of secondary metabolite produced by the plant. In that light, the chemical compositions of the same plants growing in two different regions will vary because the growth conditions of plants e.g. temperature, soil type, water and light differ from one geographical region to another.

Alkaloids, tannins, saponins, glycosides, flavonoids, and terpenoids are known antimicrobial compounds (Chandra et al., 2017). This suggests that extracts from *M. oleifera* and *X. aethiopica* may display anti-yeast activity against some fruit juice spoilage yeasts. In addition, the detected phytochemicals (Table 3.2) exhibit other biological activities such as antioxidant, anticancer, antiinflammatory, antidiabetic, antiviral, antidepressant and many others (Moosavi et al., 2018). Moreover, they are used in food to improve colour, taste and aroma (Bode and Dong, 2015).

3.4.3. Antioxidant activity of *M. oleifera* and *X. aethiopica* extracts

The DPPH assay developed by Blois (1958) is a common test for determining the antioxidant activity of plant samples. In this assay, antioxidants respond to the purple-coloured DPPH solution by donating hydrogen atom or electron and converting it to a yellow colour diphenylpicrylhydrazine molecule. The level of colour change signifies the amount and strength of antioxidants present in the compound (Lu et al., 2014).

Antioxidant activity and IC_{50} values are inversely proportional where a lower IC_{50} value indicates higher radical scavenging power (Yao et al., 2016; Alahyane et al., 2019). In this study, ascorbic acid was used as a standard antioxidant and its free radical scavenging activity (93 ± 0.156% and IC_{50} value of 2.149 µg/mL) at 15.625 µg/mL was higher than all the extracts analysed. The results indicated an increase in the free radical scavenging activities of all the plant extracts as concentration increased from 1.953 to 125 µg/mL (Figure 3.4). All the tested extracts had

considerable antioxidant activity except the water extract of *M. oleifera* which showed low activity.

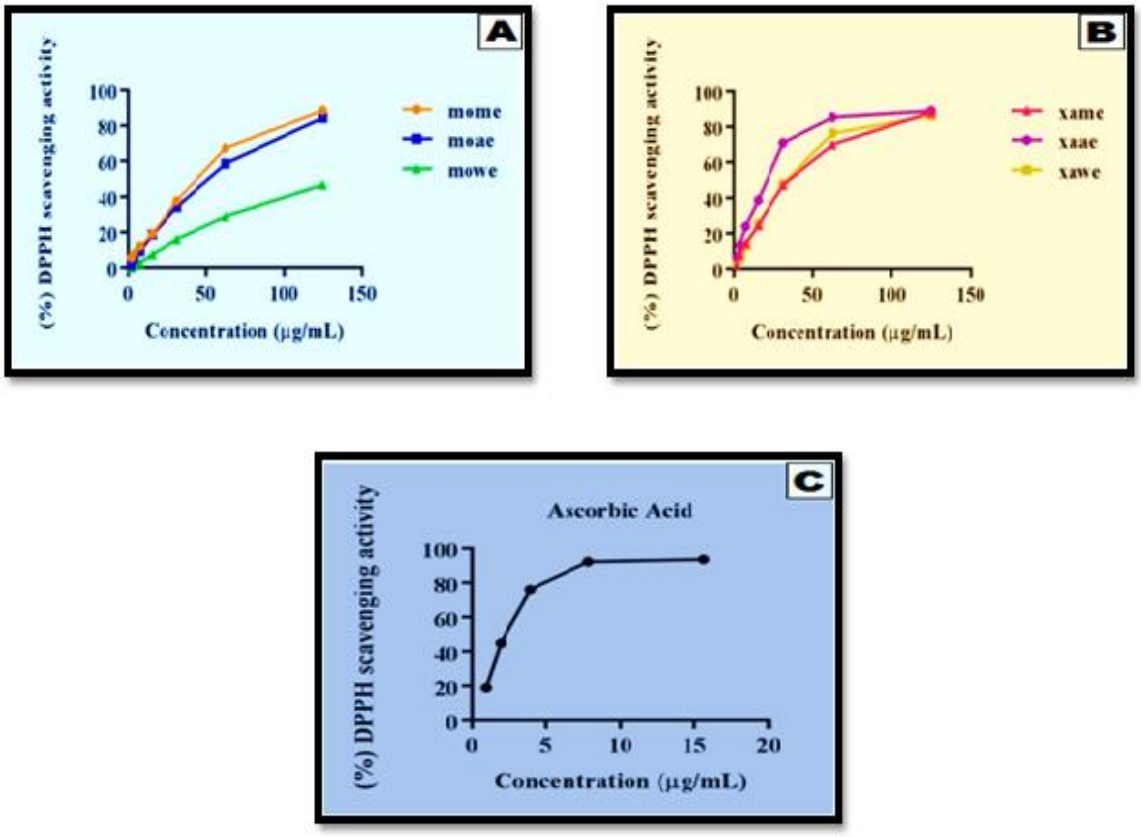

Figure 3.4. Percentage (%) DPPH radical scavenging activity of (A) *M. oleifera* extracts, (B) *X. aethiopica* extracts, (C) Ascorbic acid

The IC_{50} values of DPPH scavenging activities of plant extracts and those reported in other studies are presented in Table 3.3. Among all the extracts of *M. oleifera* analysed, the methanol extract exhibited the highest radical scavenging activity of 88.54 ± 0.509% and IC_{50} value of 48.09 ± 0.254 µg/mL. The acetone extract showed its maximum radical scavenging activity at 84.49 ± 0.544% and IC_{50} value of 86.54 ± 0.316 µg/mL. The least DPPH scavenging activity was shown by the water extract at 46.74 ± 0.667% and IC_{50} value of 124.5 ± 0.211 µg/mL. The antioxidant activity of *M. oleifera* and *X. aethiopica* extracts may be attributed to phytochemicals like flavonoids and tannins which are polyphenolic in nature (Muniyandi et al., 2019).

Table 3.3. DPPH scavenging activity of plant extracts (IC_{50} µg/mL)

	M. oleifera			*X. aethiopica*			Ascorbic Acid
	Methanol extract	**Acetone extract**	**Water extract**	**Methanol extract**	**Acetone extract**	**Water Extract**	
Results obtained from this study	48.09	86.54	124.5	31.02	19.10	40.31	2.149
Results obtained from other studies with references	**49.30** (Fitriana et al., 2016)		**46.77** (El Sohaimy et al., 2015)	**64.33** (Oso et al., 2018)	**19.82** (Mohammed and Islam, 2017)	**117.67** (Oso et al., 2018)	
	40.15 (Asgari-Kafrani et al., 2019)				**71** (Oso et al., 2018)	**280** (Ngwoke et al., 2015)	
	33.11 (El Sohaimy et al., 2015)				**620.0** (Ngwoke et al., 2015)		

Similar results were previously reported for the methanol extract of *M. oleifera*. For example, Fitriana et al. (2016) had IC_{50} of 49.30 µg/mL while Asgari-Kafrani et al. (2019) found IC_{50} of 40.15 µg/mL. The findings were, however, not consistent with El Sohaimy et al. (2015) who observed a higher antioxidant activity for the methanol extract (IC_{50} of 33.11 µg/mL) and water extract (IC_{50} of 46.77 µg/mL). This may be attributed to a number of factors such as soil type, moisture, light exposure, climate and other growth conditions in the geographical location of the plants. Bian et al. (2014) and Pérez-López et al. (2018) reported that the concentration of phenolic compounds and antioxidant activity of some plants was enhanced with increasing light intensity. This implies that plants of the same species found in different geographical locations may not have the same content and concentration of phytochemicals considering that each geographical zone is distinguished by different environmental conditions.

The acetone extract of *X. aethiopica* had the highest percentage inhibition of 89.01 ± 0.186 with an IC_{50} value of 19.10 ± 0.285 µg/mL. The methanol and water extracts showed their highest percentage inhibition at 88.15 ± 0.561 (IC_{50} value of 31.02 ± 0.123 µg/mL) and 86.45 ± 0.491 (IC_{50} value of 40.31 ± 0.157 µg/mL) respectively. The IC_{50} recorded by Oso et al. (2018) for the methanol (64.33 µg/mL), acetone (71 µg/mL) and water extract (117.67 µg/mL) from *X. aethiopica* was higher than that recorded in this study. Ngwoke et al. (2015) also observed higher IC_{50} values i.e. lower antioxidant activity for the acetone (620 µg/mL) and water extract (280 µg/mL). However, Mohammed and Islam, (2017) found a similar result for the acetone extract (IC_{50} of 19.82 µg/mL). The differences in the activity levels may be attributed to the concentration and polarity of extraction solvents used as 50% solvents were used in this study while pure solvents were used in other studies. Considering that the IC_{50} values of *X. aethiopica* extracts were lower in this study (Table 3.3), it can be inferred that hydromethanol or hydroacetone extracts more compounds with antioxidant activity from *X. aethiopica* than pure solvents.

It is important to note that the IC_{50} values of *M. oleifera* and *X. aethiopica* extracts differed from one another in this study. This supports Gharibi et al. (2015) who reported that plants exhibit different levels of antioxidant activity because they contain diverse concentrations of polyphenol. The antioxidant capacity of *M. oleifera* and *X. aethiopica* extracts qualifies them as natural inhibitors of oxidation and therefore their employment in the preservation of fruit juices may extend shelf life and improve nutritional properties. These findings indeed corroborate those in various studies that have described the use of natural antioxidants derived from plant origin such as *M. oleifera* in the fortification and preservation of food (Jayawardana et al., 2015; Falowo et al., 2017; Elhadi et al., 2017).

3.4.4. Total phenolic content of *M. oleifera* and *X. aethiopica* extracts

The total phenolic content of the extracts estimated by the Folin Ciocalteu method at 1 mg/mL was determined using the Gallic acid calibration curve in Figure 3.5.

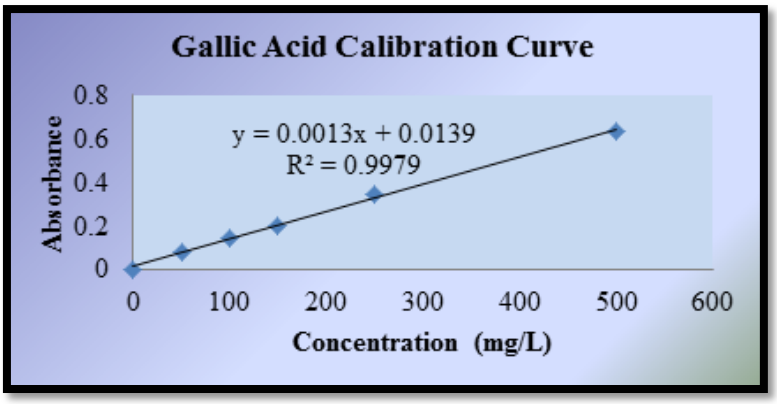

Figure 3.5. Gallic acid calibration curve for the determination of total phenolic content of *M. oleifera* and *X. aethiopica*

The highest total phenolic content for *M. oleifera* was shown by the methanol extract (110.0 ± 0.025 mg GAE/g). The acetone and water extracts showed 97.77 ± 0.060 and 68.54 ± 0.035 mg GAE/g respectively (Figure 3.6).

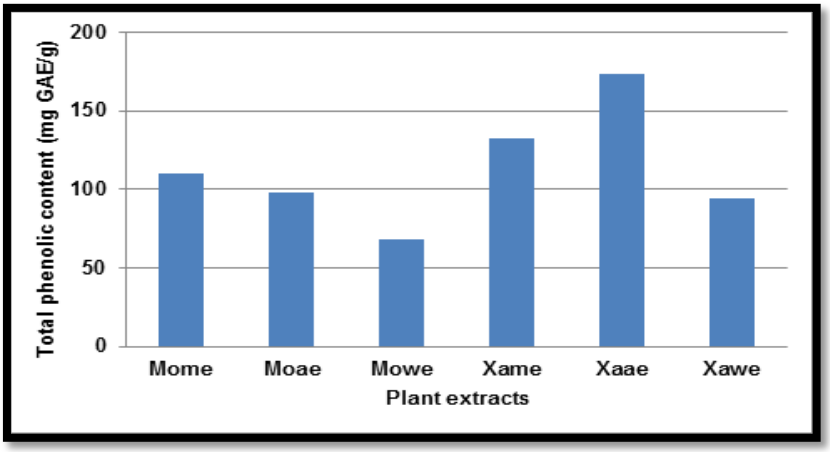

Figure 3.6. Total phenolic content of **Mome:** *M. oleifera* methanol extract, **Moae:** *M. oleifera* acetone extract, **Mowe:** *M. oleifera* water extract, **Xame:** *X. aethiopica* methanol extract, **Xaae:** *X. aethiopica* acetone extract, **Xawe:** *X. aethiopica* water extract at 1 mg/mL.

This supports the findings of El Sohaimy et al. (2015), Abdulkadir et al. (2015) and Gull et al. (2016) who reported that among all the different extracts of *M. oleifera* tested, the methanol extracts had the highest total phenolic content. The acetone extract of *X. aethiopica* had the highest total phenolic content (173.2 ± 0.043 mg GAE/g) followed by methanol extract (132.4 ± 0.020 mg GAE/g) and the least was in the water extract (93.92 ± 0.026 mg GAE/g).

As shown in Figure 3.4 and 3.6, the extracts with the strongest antioxidant activity also had the highest total phenolic content, suggesting that phenolic compounds present in the extracts of *M. oleifera* and *X. aethiopica* may be accountable for their antioxidant activity (Yao et al., 2016; Guldiken et al., 2018). Although this may not be true for other plants, Al Harthi et al. (2015) and Alahyane et al. (2019) suggest that there may be other chemical compounds besides polyphenols that are responsible for the antioxidant activity of date fruit extracts since a negative correlation was observed between the total phenolic content and antioxidant activity of date fruits. The high antioxidant activity and total phenolic content of *M. oleifera* and *X. aethiopica* extracts justifies the use of *M. oleifera* and *X. aethiopica* as medicine for different ailments (Adefegha et al., 2018; Liu et al., 2018) and as natural additives and preservatives in food (Ogbonna et al., 2013; Jayawardana et al., 2015).

3.5. Conclusion

Herbs and spices have invited significant interest over time due to their multiple functions in different industries. Their ability to impede oxidative degradation and enrich the quality and nutritional values of foods has made their use in the food industry increasingly important. This study has demonstrated that extracts from *M. oleifera* leaves and *X. aethiopica* fruits contain phenolic compounds with antioxidant activity. It is therefore necessary to perform shelf life studies on these plant extracts in order to assess their preservative potential in fruit juice. In addition, it is recommended to isolate and identify the compounds with antioxidant activity from the plants.

3.6. References

Abdulkadir, A.R., Zawawi, D.D.B., Yonusa, A.K. and Jahan, M.S. 2015. *In-vitro* antioxidant potential, total phenolic content and total flavonoid content of methanolic flower and seed extract of Miracle tree, *Moringa oleifera* Lam. *Australian Journal of Basic and Applied Sciences*, 9: 27-31.

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Al Ghasham, A., Al Muzaini, M., Qureshi, K.A., Elhassan, G.O., Khan, R.A., Farhana, S.A., Hashmi, S., El-Agamy, E. and Abdallah, W.E. 2017. Phytochemical screening, antioxidant and antimicrobial activities of methanolic extract of *Ziziphus mauritiana* Lam. leaves collected from Unaizah, Saudi Arabia. *International Journal of Pharmaceutical Research and Allied Sciences*, 6(3): 33-46.

Al Harthi, S.S., Mavazhe, A., Al Mahroqi, H. and Khan, S.A. 2015. Quantification of phenolic compounds, evaluation of physicochemical properties and antioxidant activity of four date (*Phoenix dactylifera* L.) varieties of Oman. *Journal of Taibah University Medical Sciences,* 10: 346-352.

Alahyane, A., Harrak, H., Ayour, J., Elateri, I., Ait-Oubahou, A. and Benichou, M. 2019. Bioactive compounds and antioxidant activity of seventeen Moroccan date varieties and clones (*Phoenix dactylifera* L.). *South African Journal of Botany,* 121: 402-409.

Asgari-Kafrani, A., Fazilati, M. and Nazem, H. 2019. Hepatoprotective and antioxidant activity of aerial parts of *Moringa oleifera* in prevention of non-alcoholic fatty liver disease in Wistar rats. *South African Journal of Botany*, https://doi.org/10.16/j.sajb.2019.01.014

Bian, Z.G., Yang, Q.C. and Liu, W.K. 2014. Effects of light quality on the accumulation of phytochemicals in vegetables produced in controlled

environments: A review. *Journal of the Science of Food and Agriculture,* 95(5): 869-877.

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature*, 181: 1199-1200.

Bode, A.M. and Dong, Z. 2015. Toxic phytochemicals and their potential risks for human cancer. *Cancer Prevention Research,* 8(1): 1-8.

Bower, A., Marquez, S. and de Mejia, E.G. 2016. The health benefits of selected culinary herbs and spices found in the traditional mediterranean diet. *Critical Reviews in Food Science and Nutrition*, 56(16): 2728-2746.

Braham, F., Carvalho, D.O., Almeida, C.M.R., Zaidi, F., Magalhães, J.M.C.S., Guido, L.F. and Gonçalves, M.P. 2019. Online HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from *Moringa oleifera* leaves. *South African Journal of Botany*, https://doi.org/10.1016/j.sajb.2019.04.001

Chander, M.P., Kumar, K.V., Shriram, A.N. and Vijayachari, P. 2015. Antileptospiral activities of an endemic plant *Glyptopetalum calocarpum* (Kurz.) Prain used as a medicinal plant by Nicobarese of Andaman and Nicobar Islands. *Natural Product Research*, 29(16): 1575-1577.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A.P. and Nautiyal, A.R. 2017. Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials: A review. *Plants,* 6: 16.

Ekeanyanwu, R.C. and Etienajirhevwe, O.F. 2012. *In vitro* anthelmintic potentials of *Xylopia aethiopica* and *Monodora myristica* from Nigeria. *African Journal of Biochemistry Research,* 6: 115-120.

Ekeh F.N., Odo, G.E., Nzei, J., Ngozi, E., Ohanu, C. and Onuoha, O. 2015. Efficacy of *Xylopia aethiopica* ethanolic and aqueous extracts on the control of *Sitophilus oryzae* in stored rice grain. *African Journal of Agricultural Research*, 13(10): 470-476.

El Sohaimy, S.A., Hamad, G.M., Mohamed, S.E., Amar, M.H. and Al-Hindi, R.R. 2015. Biochemical and functional properties of *Moringa oleifera* leaves and

their potential as a functional food. *Global Advanced Research Journal of Agricultural Science*, 4: 188-199.

Elhadi, D.A.E., Elgasim, E.A. and Ahmed, I.A.M. 2017. Microbial and oxidation characteristics of refrigerated chicken patty incorporated with moringa (*Moringa oleifera*) leaf powder. *CyTA - Journal of Food*, 15(2): 234-240.

Ephrem, E., Najjar, A., Charcosset, C. and Gergesa, H.G. 2018. Encapsulation of natural active compounds, enzymes, and probiotics for fruit juice fortification, preservation, and processing: An overview. *Journal of Functional Foods*, 48: 65-84.

Falowo, A.B., Muchenje, V., Hugo, A., Aiyegoro, O.A. and Fayemi, P.O. 2017. Antioxidant activities of *Moringa oleifera* L. and *Bidens pilosa* L. leaf extracts and their effects on oxidative stability of ground raw beef during refrigeration storage. *CyTA - Journal of Food,* 15(2): 249-256.

Fitriana, W. D., Ersam, T., Shimizu, K. and Fatmawati, S. 2016. Antioxidant activity of *Moringa oleifera* extracts. *Indonensian Journal of Chemistry*, 16(3): 297-301.

Gharibi, S., Tabatabaei, B.E.S. and Saeidi, G. 2015. Comparison of essential oil composition, flavonoid content and antioxidant activity in eight *Achillea* species. *Journal of Essential Oil Bearing Plants,* 18: 1382-1394.

Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F.D., Yalcinkaya, I.E. and Capanoglu, E. 2018. Phytochemicals of herbs and spices: health versus toxicological effects. *Food and Chemical Toxicology,* 119: 37-49.

Gull, I., Javed, A., Aslam, M.S., Mushtaq, R. and Athar, M.A. 2016. Use of *Moringa oleifera* flower pod extract as natural preservative and development of SCAR marker for its DNA based identification. *BioMed Research International,* 2016: 1-12.

Haneen, H.S.M. 2015. Effect of dried *Moringa oleifera* leaves on the nutritional and organoleptic characteristics of cookies. *Alexandria Science Exchange Journal,* 36(4): 297-305.

Harsha, N., Sridevi, V., Lakshmi, M., Rani, K. and Vani, K. 2013. Phytochemical analysis of some selected spices. *International Journal of Innovative Research in Science, Engineering and Technology*, 2(11): 6618-6621.

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S. and Weththasinghe, P. 2015. Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. *LWT - Food Science and Technology*, 64: 1204-1208.

Khatua, S., Ghosh, S. and Acharya, K. 2017c. Simplified methods for micro-titer based analysis of *in vitro* antioxidant activity. *International Journal of Research in Pharmaceutical Sciences,* 11(2): S327-S335.

Khazdair, M.R., Ghorani, V., Alavinezhad, A. and Boskabady, M.H. 2018. Pharmacological effects of *Zataria multiflora* Boiss L. and its constituents focus on their anti-inflammatory, antioxidant, and immunomodulatory effects**.** *Fundamental and Clinical Pharmacology*, 32(1): 26-50.

Kuikman, M. and O'Connor, C.P. 2015. Sensory evaluation of moringa-probiotic yogurt containing banana, sweet potato or avocado. *Journal of Food Research*, 4: 165-171.

Kumar, S., Yadav, A., Yadav, M and Yadav, J.P. 2017. Effect of climate change on phytochemical diversity, total phenolic content and *in vitro* antioxidant activity of *Aloe vera* (L.) Burm.f. *BMC Research Notes,* 10(1): 60.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

Lu, Y., Khoo, T.J. and Wiart, C. 2014. Antioxidant activity determination of citronellal and crude extracts of *Cymbopogon citratus* by 3 different methods. *Pharmacology and Pharmacy,* 5: 395-400.

Maqsood, M., Qureshi, R., Arshad, M., Ahmed, M.S. and Ikram, M. 2017. Preliminary phytochemical screening, antifungal and cytotoxic activities of leaves extract of *Moringa oleifera* Lam. from salt range, Pakistan. *Pakistan Journal of Bot*any, 49(1): 353-359.

Mohammed, A. and Islam, M.S. 2017. Antioxidant potential of *Xylopia aethiopica* fruit acetone fraction in a type 2 diabetes model of rats. *Biomedicine and Pharmacotherapy,* 96: 30-36.

Moosavi, M.A., Haghi, A., Rahmati, M., Taniguchi, H., Mocan, A., Echeverría, J., Gupta, V.K., Tzvetkov, N.T. and Atanasov, A.G. 2018. Phytochemicals as potent modulators of autophagy for cancer therapy. *Cancer Letters*, 424: 46-69.

Muniyandi, K., George, E., Sathyanarayanana, S., George, B.P., Abrahamse, H., Thamburaj, S. and Thangaraj, P. 2019. Phenolics, tannins, flavonoids and anthocyanins contents influenced antioxidant and anticancer activities of Rubus fruits from Western Ghats, India. *Food Science and Human Wellness,* 8: 73-81.

Ngo, T.V., Scarlett, C.J., Bowyer, M.C., Ngo, P.D. and Vuong, Q.V. 2017. Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *Journal of Food Quality,* 2017: 1-8.

Ngwoke, K.G., Ikeanyi, A.U., Eze, P.M., Ezemokwe, I.C., Abba, C.C. and Ugwu, M.C. 2015. Phytochemical and antioxidant properties of extracts of *Xylopia aethiopica* fruits. *Chemical Science Review and Letters*, 4(13): 267-270.

Ogbonna C.N., Nozaki, K. and Yajima, H. 2013. Antimicrobial activity of *Xylopia aethiopica*, *Aframomum melegueta* and *Piper guineense* ethanolic extracts and the potential of using *Xylopia aethiopica* to preserve fresh orange juice. *African Journal of Biotechnology,* 12(16): 1993-1998.

Oso, B.J., Oyeleke, O. and Soetan, O. 2018. Influence of different solvent polarities on the phenolics, flavonoids and antioxidant properties of the fruit of *Xylopia aethiopica* (Dunal) A. Rich. *Trends in PhytochemIcal Research*, 2(2): 97-102.

Padmalochana, K. 2018. Antiinflammatory activity and phytochemical analysis of *Moringa oleifera* ethanol and acetone leaves extract. *Journal of Drug Delivery and Therapeutics*, 8: 269-273.

Patel, N., Patel, P., Patel, D., Desai, S. and Meshram, D. 2014. Phytochemical analysis and antibacterial activity of *Moringa oleifera. International Journal of Medicine and Pharmaceutical Sciences,* 4(2): 27-34.

Peña-Bautista, C., Baquero, M., Vento, M. and Cháfer-Pericás, C. 2019. Free radicals in Alzheimer's disease: Lipid peroxidation biomarkers. *Clinica Chimica Acta,* 491: 85-90.

Pérez-López, U., Sgherri, C., Miranda-Apodaca, J., Micaelli, F., Lacuesta, M., Mena-Petite, A. and Muñoz-Rueda, A. 2018. Concentration of phenolic compounds is increased in lettuce grown under high light intensity and elevated CO_2. *Plant Physiology and Biochemistry,* 123: 233-241.

Saeed, N., Khan, M.R. and Shabbir, M. 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complementary and Alternative Medicine,* 12: 221.

Sepahpour, S., Selamat, J., Abdul Manap, M.Y., Khatib, A. and Abdull Razis, A.F. 2018. Comparative analysis of chemical composition, antioxidant activity and quantitative characterization of some phenolic compounds in selected herbs and spices in different solvent extraction systems. *Molecules*, 23: 402.

Shanmugavel, G., Prabakaran, K. and George, B. 2018. Evaluation of phytochemical constituents of *Moringa oleifera* (Lam.) leaves collected from Puducherry region, South India. *International Journal of Zoology and Applied Biosciences*, 3(1): 1-8.

Silva, M.M. and Lidon, F.C. 2016. An overview on applications and side effects of antioxidant food additives. *Emirates Journal of Food and Agriculture*, 28(12): 823-832.

Sokamte, T.A., Mbougueng, P.D., Tatsadjieu, N.L. and Sachindra, N.M. 2019. Phenolic compounds characterization and antioxidant activities of selected spices from Cameroon. *South African Journal of Botany,* 121: 7-15.

Souza, V.G.L., Rodrigues, P.F., Duarte, M.P. and Fernando, A.L. 2018. Antioxidant migration studies in chitosan films incorporated with plant extracts. *Journal of Renewable Materials*, 6(5): 548-558.

Tzima, K., Makris, D., Nikiforidis, C.V. and Mourtzinos, I. 2015. Potential use of rosemary, propolis and thyme as natural food preservatives. *Journal of Nutrition and Health*, 1: 6.

Vallverdú-Queralt, A., Regueiro, J., Martínez-Huélamo, M., Alvarenga, J.F.R., Leal, L.N. and Lamuela-Raventos, R.M. 2014. A comprehensive study on the phenolic profile of widely used culinary herbs and spices: Rosemary, thyme, oregano, cinnamon, cumin and bay. *Food Chemistry*, 154: 299-307.

Vinoth, B., Manivasagaperumal, R. and Balamurugan, S. 2012. Phytochemical analysis and antibacterial activity of *Moringa oleifera* Lam. *International Journal of Research in Biological Sciences,* 2(3): 98-102.

Vladimir-Knežević, S., Blažeković, B., Štefan, M.B., Alegro, A., Kőszegi, T. and Petrik, J. 2011. Antioxidant activities and polyphenolic contents of three selected *Micromeria* species from Croatia. *Molecules,* 16: 1454-1470.

Waterhouse, A.L. 2003. Determination of total phenolics. In: *Current protocols in food analytical chemistry*. Wrolstad, R.E., Ed. New York, USA: John Wiley and Sons. pp. 111-118.

Yang, L., Wen, K.S., Ruan, X., Zhao, Y.X., Wei, F. and Wang, Q. 2018. Response of plant secondary metabolites to environmental factors. *Molecules*, 23(4): 762.

Yao, X.H., Zhang, Z.B., Song, P., Hao, J.Y., Zhang, D.Y. and Zhang, Y.F. 2016. Different harvest seasons modify bioactive compounds and antioxidant activities of *Pyrola incarnata. Industrial Crops and Products,* 94: 405-412.

Chapter Four

Anti-yeast activity of *Moringa oleifera* and *Xylopia aethiopica* extracts and their potential as natural preservatives in fruit juice

4.1. Abstract

Most fruit juice preservatives are chemically synthesised, and knowledge of the health risks associated with their consumption has resulted in the search for safer alternatives, such as secondary plant metabolites. This study evaluated the anti-yeast properties of methanol, acetone and water extracts from *Moringa oleifera* and *Xylopia aethiopica* as well as their antimicrobial preservative potential in fresh grape and orange juice. The extracts were tested against ten yeast isolates involved in fruit juice spoilage using the agar dilution assay. All the extracts of *M. oleifera* stimulated the growth of *Yarrowia lipolytica* and inhibited *Rhodotorula dairenensis* at 0.625 mg/mL. The methanol extract was fungicidal against *Wickerhamomyces anomalus* at 10 mg/mL, the CY0757 strain of *Zygosaccharomyces bailii* at 2.5 mg/mL and the IGC4242 strain at 5 mg/mL. The acetone and water extracts exhibited the least anti-yeast activity. *Lodderomyces elongisporus, Saccharomyces cerevisiae* ATCC26602 and IGC3507, *Cryptococcus laurentii* and *Candida parapsilosis* were the least affected by *M. oleifera* extracts. The methanol extract of *X. aethiopica* was fungicidal against *Z. bailii* IGC4242 at 2.5 mg/mL and *C. laurentii* at 5 mg/mL. Although the acetone extract was not fungicidal against any of the yeasts, it showed inhibitory activity against most of them. The water extract was the least active of all extracts. *C. parapsilosis, S. cerevisiae* ATCC26602 and IGC3507 were the most insensitive yeast to *X. aethiopica* extracts. Microbiological analysis of treated fruit juices showed overgrowth after 3 days of storage. This study concludes that *M. oleifera* and *X. aethiopica* extracts have limited potential as antimicrobial preservatives in fruit juice. However, the growth stimulatory activity of *M. oleifera* extracts on *Yarrowia lipolytica* may be useful in the production of biofuels and warrants further investigation.

Keywords: Beverage, yeast spoilage, secondary plant metabolites, antimicrobials, fruit juice preservative.

4.2. Introduction

Nutritious and ready-to-serve beverages such as fruit juices have increasingly become a household drink in many parts of the world due to their richness in sugars, vitamins, polyphenols and other bioactive compounds that have health promoting properties. Nevertheless, their bountiful nutrients and high sugar content are seedbeds for microbial growth thus making them highly susceptible to spoilage (Cardador and Gallego, 2015; Soto et al., 2019). A variety of microorganisms such as acid tolerant bacteria (*Acetobacter, Alicyclobacillus, Lactobacillus, Leuconostoc, Gluconobacter, Bacillus, Zymomonas* and *Zymobacter*), yeasts (*Saccharomyces, Rhodotorula, Candida, Pichia, Zygosaccharomyces*, etc.) and moulds (*Penicillium, Cladosporium, Botrytis, Aspergillus, Aureobasidium,* etc.) can cause spoilage in fruit juices. However, yeasts are the primary contaminants of fresh and concentrated fruit juices, mainly because many of them can grow anaerobically and in high acidic environments (Bevilacqua et al., 2012; Aneja et al., 2014).

The activity of yeasts in fruit juice often results in off-flavour, off-odour, flocculation, turbidity, enhanced production of carbon dioxide and phase separation through the action of enzymes on the pectin, all of which lead to loss of product quality and ultimately waste (Maciel et al., 2013; Bukvicki et al., 2014). The presence of contaminants, especially yeasts and moulds in finished juice products, have been reported by many juice producers who also believe that profit could rise by 92% and waste reduced by 64% if microbial spoilage was scientifically addressed in the food and beverage industry (Snyder and Worobo, 2018; Biango-Daniels et al., 2019).

Specifically, osmophilic and acid-tolerant yeasts from the *Zygosaccharomyces* genus are frequently isolated from spoiled foods and beverages with high sugar concentration due to their peculiar abilities to withstand weak acid preservatives, ferment hexose sugar and thrive at low pH (Wang et al., 2016). *Zygosaccharomyces* are accountable for the huge economic losses experienced by juice producers and therefore are regarded as major threats to the industry (Hernández et al., 2018).

Over the years, thermal processing such as pasteurisation and the use of chemical preservatives have been conventionally used to maintain the quality and safety of fruit juices (Guedes et al., 2016). Even so, their applications are hindered by different drawbacks. For example, changes in the sensory qualities (aroma, flavour and colour) and loss of nutritional properties (vitamins, carotenoids, polyphenols, etc.) may occur in fruit juices that have been subjected to heat treatment. Likewise, studies have reported that constant exposure to chemical compounds e.g. sulphur dioxide, benzoic and sorbic acids used in the preservation of fruit juices may produce allergic, neurotoxic, carcinogenic and other adverse effects on human health (Guedes et al., 2016; Ng et al., 2019). As a result, different approaches such as ultrasound, pulsed electric field, UV-C light as well as the application of natural compounds from plant sources are continuously being researched as alternatives in ensuring the safety and quality of fruit juices (Almeida et al., 2019).

Plants like *M. oleifera* and *X. aethiopica* contain a variety of secondary metabolites with numerous health-promoting characteristics such as antiulcer, antidiabetic, antiproliferative, antimicrobial, antiviral, antiinflammatory, anticancer, antioxidant, antidepressant and so much more (Adefegha et al., 2018; Liu et al., 2018). Chapter three of this study has confirmed that extracts from *M. oleifera* and *X. aethiopica* exhibit high antioxidant activity which may play a part in the preservation of fruit juice. Moreover, studies have demonstrated their potential use as natural preservatives in food (Ogbonna et al., 2013; Jayawardana et al., 2015). This study was conducted to explore the anti-yeast activity of *M. oleifera* and *X. aethiopica* extracts and their potential use as natural substitutes for synthetic preservatives in grape and orange juice.

4.3. Methods

4.3.1. *In vitro* anti-yeast activity of *M. oleifera* and *X. aethiopica* extracts

The extracts of *M. oleifera* and *X. aethiopica* were prepared from ground plant material as described in Chapter Three (section 3.3.1). The anti-yeast activity of the

extracts was determined using the agar dilution method according to López-Carballo et al. (2012).

4.3.1.1. Yeast and culture conditions

Ten different yeasts involved in fruit juice spoilage were used in this study. *Lodderomyces elongisporus*, *Wickerhamomyces anomalus, Candida parapsilosis, Yarrowia lipolytica*, *Rhodotorula dairenensis* and *Cryptococcus laurentii* were obtained from the Centre for Applied Food Sustainability and Biotechnology (CAFSaB) Central University of Technology, Free State culture collection. These specimens were isolated from the production environment and spoiled product of a fruit juice production facility (Corbett and de Smidt, 2019), *Zygosaccharomyces bailii* CY0757 and IGC4242 and *Saccharomyces cerevisiae* ATCC26602 and IGC3507 were sourced from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free State. The yeast cultures were maintained at -20^{o}C in Yeast extract Peptone Dextrose (YPD) broth containing 15% glycerol.

4.3.1.2. Integration of plant extracts into medium

The plant extracts were dissolved in 30 mL of sterile distilled water, 1% acetone and 1% methanol to make an initial concentration of 20, 10, 5, 2.5 and 1.25 mg/mL. Subsequently, 30 mL of warm molten Potato Dextrose Agar (PDA) was added to the extracts and gently swirled. The mixture with a final concentration of 10, 5, 2.5, 1.25 and 0.625 mg/mL was poured into petri dishes and allowed to solidify. A set of controls consisting of a blank (only PDA) and PDA mixed with 1% acetone and 1% methanol were prepared in order to ensure the non-inhibitory effect of the solvents at 1% against the yeasts. All experiments were carried out aseptically in the laminar flow hood and each test was performed in triplicate.

4.3.1.3. Preparation of Inocula

Yeast cultures were revived by sub-culturing on PDA plates and incubating at 28^{o}C for 48 hours. After incubation, a sterile loop was used to transfer well isolated colonies onto fresh PDA plates and incubated for a further 48 hours. This was done

to ensure that the colonies obtained were of pure cultures. Thereafter, cells from the 48 hour culture were added directly into 5 mL of sterile saline, standardized to 0.5 McFarland's turbidity with a spectrophotometer (Spectramax *M2*^{e}) and inoculated into 20 mL of broth containing glucose (0.4 g) and peptone (0.2 g). The inoculated broth was then loaded onto a MAST Uri®Dot multipoint inoculator (Figure 4.1) and cells were immediately distributed to the PDA plates at various points.

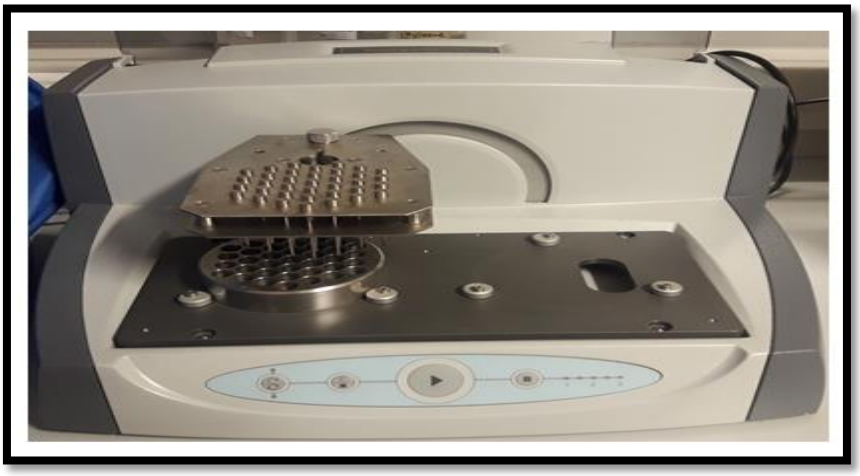

Figure 4.1. A multipoint inoculator

The plates were incubated at 28^{o}C and yeast growth was recorded after 48, 72 and 120 hours. The anti-yeast activity of the plant extracts was determined by measuring the diameter (mm) of yeast colonies with a Central 6420 Vernier Calliper and expressed as percentage growth inhibition using the formula below. Minimum inhibitory concentrations (MIC) were documented at 48 hours of treatment.

$$\% \text{ inhibition} = \frac{\text{growth of yeast in control} - \text{growth of yeast in extract}}{\text{growth of yeast in control}} \text{ x } 100$$

4.3.1.4. Statistical analysis

Data for anti-yeast activity was subjected to analysis of variance (ANOVA) using SAS software version 9.1, where differences between treatment means were ranked using the Least Significance Difference test (LSD 0.05).

4.3.2. Preparation of grape and orange juice

Fresh oranges and white grapes were purchased from Food Lovers' Market in Bloemfontein and transported to the laboratory. The fruits were thoroughly rinsed with sterile distilled water and orange peels were removed. Thereafter, fruit juices were extracted using a juice extractor (Figure 4.2) and passed through sterile Whatman No. 1 filter paper in order to remove the fibres. The fruit juices were stored in clean sterile bottles and kept in the refrigerator at 4^{o}C for 18 hours.

Figure 4.2. Preparation of fruit juice using a juice extractor.

4.3.3. Anti-yeast activity of *M. oleifera* and *X. aethiopica* extracts in fresh fruit juice

The plant extracts and concentrations used in this experiment were selected based on their fungicidal effect against *C. laurentii*, the IGC4242 and CY0757 strains of *Z. bailii* (Table 4.1 and 4.2). The potential of the methanol extract of *M. oleifera* and *X. aethiopica* as natural antimicrobials in fruit juices was therefore evaluated using the following procedure: Fresh grape and orange juice (270 mL) were separately used to dissolve 2.7 g and 1.35 g of the methanol extract from *M. oleifera* and *X. aethiopica* to give a final concentration of 10 and 5 mg/mL respectively. The mixtures were constantly stirred on a magnetic stirrer until the extracts were completely dissolved. Afterwards, 45 mL of the treated fruit juices were dispensed into separate plastic centrifugal tubes (Figure 4.3).

C. laurentii and *Z. bailii* IGC4242 and CY0757 were grown in a broth consisting of glucose and peptone at 28^{o}C. To ensure the yeasts were in their exponential phases before inoculating them into the fruit juices, their growth was monitored every two hours (up to 12 hours for *C. laurentii*, 16 hours for the CY0757 strain of *Z. bailli* and 18 hours for the IGC4242 strain) using a spectrophotometer (Spectramax *M2*^{e}), where the optical densities were plotted against time (hours).

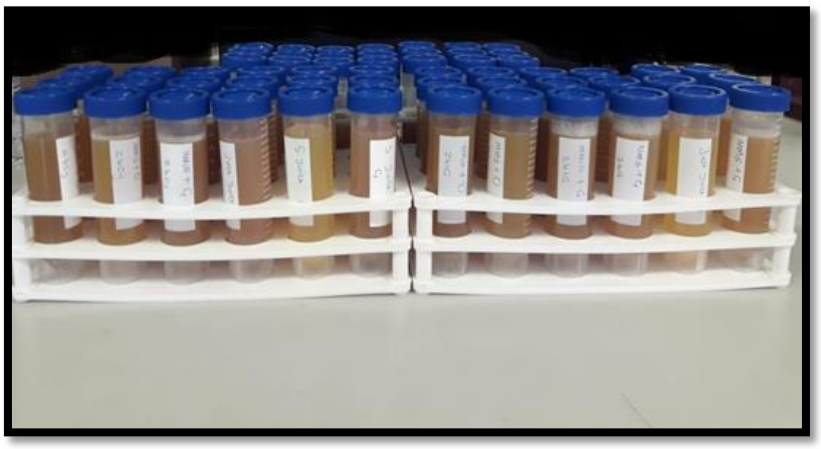

Figure 4.3. Grape and orange juice incorporated with plant extracts

Thereafter, 100 µL of the yeasts were diluted in 9 mL of sterile saline solution (9 g/L NaCl) and fruit juices treated with the methanol extract of *M. oleifera* were inoculated with 100 µL of diluted *Z. bailii* CY0757 and IGC4242 while those treated with the methanol extract of *X. aethiopica* were inoculated with 100 µL of diluted *C. laurentii* and the IGC4242 strain of *Z. bailii*. Fresh grape and orange juice without extracts served as controls and all samples were kept at room temperature for three days.

4.3.4. Microbiological analysis of treated fruit juices

The inhibitory effect of the plant extracts on the spoilage yeasts inoculated into the grape and orange juice was evaluated using the spread plate method and counting the viable cells present (Bukvicki et al., 2014). One millilitre of treated fruit juices was diluted with 9 mL of sterile saline (9 g/L NaCl) and 100 µL of the mixture was plated on PDA and incubated at 28^{o}C for 48 hours.

4.4. Results and discussion

4.4.1. *In vitro* anti-yeast activity of *M. oleifera* and *X. aethiopica* extracts

The agar dilution assay is a common method used to determine the minimum concentration a compound with antimicrobial activity requires to kill or inhibit microorganisms. This assay allows multiple compounds to be tested at the same time and easy detection of contaminants (López-Carballo et al., 2012).

The anti-yeast activity of *M. oleifera* and *X. aethiopica* extracts as percentage growth inhibition of *W. anomalus, Z. bailii* CY0757 and IGC4242, *R. dairenensis, Y. lipolytica, L. elongisporus, C. laurentii, C. parapsilosis* and *S. cerevisiae* ATCC26602 and IGC3507 are presented in Table 4.1 and 4.2 respectively. The plant extracts revealed different levels of activity against the yeasts at various time intervals (48, 72 and 120 hours) and concentrations (10 - 0.625 mg/mL). Acetone and methanol at 1% were not significantly different from the controls used in this study and they were not inhibitory to any of the tested yeasts.

As shown in Table 4.1, *L. elongisporus, C. laurentii, C. parapsilosis, S. cerevisiae* ATCC26602 and IGC3507 were the least susceptible yeasts to *M. oleifera* with maximum inhibition of 38.3% after 120 hours. The methanol extract in particular, showed the highest inhibitory activity (100%) against *W. anomalus* at 5 mg/mL within the 48 -hour period. But, as time progressed to 72 and 120 hours, the extract was seen to inhibit the yeast by 47.1 and 32.9% respectively. This implies that the methanol extract was fungistatic at 5 mg/mL and fungicidal at 10 mg/mL against *W. anomalus*. Similarly, the methanol extract exerted 100% inhibition against the CY0757 and IGC4242 strain of *Z. bailii* throughout the investigated time periods. However, after 120 hours the extract showed 73.6% inhibition against the IGC4242 strain at 2.5 mg/mL, suggesting that the methanol extract was fungistatic at 2.5 mg/mL and fungicidal at 5 mg/mL against the IGC4242 strain of *Z. bailii*. The methanol extract further showed a concentration-dependent inhibitory activity ranging from 32.8 - 56.9% after 48 hours, 50.6 - 68.2% after 72 hours and 50.3 - 57.9% after 120 hours and MIC of 0.625 mg/mL against *R. dairenensis*. Bioactivity

against *R. dairenensis* at 48 and 72 hours was not significantly different. Interestingly, *Y. lipolytica* thrived in the presence of the methanol extract as time and concentration increased (Figure 4.4).

The acetone extract of *M. oleifera* displayed less inhibitory activity compared to the methanol extract. This finding contradicts Dzoyem et al. (2016), who described acetone as the best solvent for extracting antimicrobial compounds from plants. The result from this study may be attributed to the acetone concentration (50%) that was used to extract the plant material. It is possible that the anti-yeast compounds in *M. oleifera* leaves were not soluble in 50% acetone. As with the methanol extract, the acetone extract showed growth stimulatory activity on *Y. lipolytica* with increasing concentration and time. It is worth noting that the yeast behaved slightly differently toward the acetone extracts in the sense that it showed resistance at 5 mg/mL but as concentration decreased from 2.5 mg/mL the increase in yeast size became notable (Figure 4.5).

Liu et al. (2018) reported that *M. oleifera* leaves are enriched with unsaturated fatty acids. This might explain the stimulatory effect exhibited by the extracts on *Y. lipolytica*, keeping in mind that the aerobic, dimorphic yeast utilises hydrophobic substrates such as *n*-alkanes, fats, oils and fatty acids as carbon sources (Gonçalves et al., 2014). Nevertheless, further study is required to confirm the actual compound(s) in *M. oleifera* responsible for enhancing the growth of *Y. lipolytica*; a useful yeast in biofuel production (Darvishi et al., 2017).

Besides enhancing the growth of *Y. lipolytica* and showing inhibitory activity against *R. dairenensis* (Figure 4.6), the water extract was a poor growth inhibitor against the tested yeasts. This can be expected as organic solvents are more effective in extracting antimicrobial compounds from plants than water. Water being a polar solvent rarely shows antimicrobial activity (Dzoyem et al., 2016).

Table 4.1. Growth inhibition of yeast (%) after treating with *M. oleifera* extracts at different time intervals

Extract	Time (hours)	Concentration (mg/mL)	WA	ZB1	ZB2	RD	YL	LE	CL	CP	SC1	SC2
Methanol	48	10	100.0^{p}	100.0^{n}	100.0^{m}	56.9^{q}	-75.5^{on}	**6.9^{f}**	9.1^{f}	10.0^{l}	19.4^{j}	38.3^{l}
		5	**100.0^{p}**	100.0^{n}	100.0^{m}	50.0^{p}	-55.1^{qr}	0.0^{e}	0.0^{e}	**10.0^{l}**	**19.4^{j}**	27.7^{k}
		2.5	0.0^{n}	100.0^{n}	100.0^{m}	37.9^{on}	-51.0^{qrs}	0.0^{e}	0.0^{e}	0.0^{k}	11.1^{ji}	**14.9^{j}**
		1.25	0.0^{n}	42.5^{m}	20.6^{kj}	37.9^{on}	-42.9^{tus}	0.0^{e}	0.0^{e}	0.0^{k}	11.1^{jl}	2.1^{l}
		0.625	0.0^{n}	**32.5^{kl}**	**14.7^{ij}**	**32.8^{mln}**	-36.7^{u}	0.0^{e}	0.0^{e}	0.0^{k}	0.0^{i}	2.1^{i}
		1% methanol	0.0^{n}	5.0^{gf}	0.0^{gf}	1.7^{ij}	2.0^{y}	0.0^{e}	0.0^{e}	0.0^{k}	5.6^{i}	2.1^{i}
		control	0.0^{n}	0.0^{gf}	0.0^{gf}	0.0^{ij}	0.0^{y}	0.0^{e}	0.0^{e}	0.0^{k}	0.0^{i}	0.0^{i}
	72	10	100.0^{p}	100.0^{n}	100.0^{m}	68.2^{qp}	-106.8^{cd}	13.0^{d}	15.8^{l}	22.8^{j}	16.4^{h}	34.4^{j}
		5	47.1^{o}	100.0^{n}	100.0^{m}	65.9^{p}	-78.0^{hg}	13.0^{d}	10.5^{ih}	17.5^{i}	14.5^{gh}	23.0^{ih}
		2.5	25.0^{ml}	100.0^{n}	100.0^{m}	54.1^{mln}	-78.0^{hg}	13.0^{d}	10.5^{ih}	10.5^{hg}	10.9^{gth}	23.0^{ih}
		1.25	25.0^{ml}	13.0^{gf}	31.0^{lj}	54.1^{mln}	-67.8^{jl}	13.0^{d}	10.5^{ih}	10.5^{hg}	7.3^{gf}	13.1^{gf}
		0.625	19.1^{kj}	13.0^{gf}	19.0^{gf}	50.6l	-39.0^{op}	13.0^{d}	5.3^{gh}	10.5^{hg}	7.3^{gf}	9.8^{gf}
		1% methanol	1.5^{de}	0.0^{d}	0.0^{ed}	1.2^{b}	1.7^{wv}	0.0^{c}	2.6^{g}	0.0^{e}	3.6^{ed}	3.3^{cd}
		control	0.0^{de}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}	0.0^{c}	0.0^{gf}	0.0^{e}	0.0^{ed}	0.0^{cb}
	120	10	100.0^{p}	100.0^{n}	100.0^{m}	57.9^{gh}	-120.3^{a}	16.4^{c}	19.0^{c}	24.3^{fg}	27.9^{gfh}	28.2^{gh}
		5	32.9^{ml}	100.0^{n}	100.0^{m}	53.8^{fe}	-73.9^{d}	7.3^{b}	19.0^{c}	18.6^{e}	17.6^{ed}	16.9^{cd}
		2.5	19.7^{gh}	100.0^{n}	73.6^{l}	53.8^{fe}	-73.9^{d}	7.3^{b}	19.0^{c}	18.6^{e}	16.2^{d}	11.3^{b}
		1.25	11.8^{de}	16.7^{d}	15.1^{cb}	50.3^{c}	-65.2^{e}	7.3^{b}	17.5^{c}	12.9^{d}	16.2^{d}	11.3^{b}
		0.625	9.2^{dc}	14.8^{d}	13.2^{b}	50.3^{c}	-29.0^{mn}	7.3^{b}	15.9^{c}	10.0^{cd}	16.2^{d}	2.8^{a}
		1% methanol	1.3^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}	0.0^{a}	3.2^{a}	0.0^{a}	0.0^{a}	0.0^{a}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Acetone	48	10	0.0^{n}	37.5^{ml}	26.5^{k}	50.0^{p}	22.4^{z}	0.0^{e}	**10.5^{ih}**	0.0^{k}	19.4^{j}	10.6^{j}
		5	0.0^{n}	27.5^{kj}	20.6^{kj}	41.4^{o}	14.3^{z}	0.0^{e}	5.3^{gh}	0.0^{k}	**19.4^{j}**	**10.6^{j}**
		2.5	0.0^{n}	27.5^{kj}	14.7^{ij}	37.9^{on}	2.0^{y}	0.0^{e}	5.3^{gh}	0.0^{k}	11.1^{ji}	4.3^{i}
		1.25	0.0^{n}	27.5^{kj}	**14.7^{ij}**	34.5^{mn}	-20.4^{wv}	0.0^{e}	5.3^{gh}	0.0^{k}	11.1^{ji}	2.1^{i}
		0.625	0.0^{n}	**25.0^{ij}**	5.9^{gh}	**34.5^{mn}**	-36.7^{u}	0.0^{e}	2.6^{g}	0.0^{k}	5.6^{i}	0.0^{ih}
		1% acetone	0.0^{n}	5.0^{gf}	5.9^{gh}	1.7^{ij}	2.0^{y}	0.0^{e}	0.0^{gf}	0.0^{k}	0.0^{i}	0.0^{ih}
		control	0.0^{n}	0.0^{gf}	0.0^{gh}	0.0^{ij}	0.0^{y}	0.0^{e}	0.0^{gf}	0.0^{k}	0.0^{i}	0.0^{ih}
	72	10	19.1^{kj}	41.3^{kl}	28.6^{ih}	60.0^{o}	13.6^{yx}	13.0^{d}	13.0^{f}	14.0^{hi}	14.5^{gh}	13.1^{gf}
		5	16.2^{ij}	34.8^{ij}	19.0^{gf}	57.6^{on}	6.8^{wx}	13.0^{d}	0.0^{ed}	10.5^{hg}	14.5^{gh}	9.8^{ef}
		2.5	16.2^{ij}	13.0^{gf}	19.0^{gf}	55.3^{mn}	-22.0^{trs}	13.0^{d}	0.0^{ed}	10.5^{hg}	7.3^{gf}	6.6^{ed}
		1.25	7.4^{gf}	13.0^{gf}	14.3^{f}	50.6^{l}	-42.4^{o}	13.0^{d}	0.0^{ed}	10.5^{hg}	0.0^{ed}	6.6^{ed}
		0.625	2.9^{de}	8.7^{ef}	14.3^{f}	50.6^{l}	-45.8^{on}	13.0^{d}	0.0^{ed}	0.0^{e}	0.0^{ed}	0.0^{cb}
		1% acetone	1.5^{de}	0.0^{d}	0.0^{ed}	1.2^{b}	1.7^{wv}	0.0^{c}	0.0^{ed}	0.0^{e}	0.0^{ed}	0.0^{cb}
		control	0.0^{de}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}	0.0^{c}	0.0^{ed}	0.0^{e}	0.0^{ed}	0.0^{cb}

	120	10	14.5^{fe}	46.3^{kj}	22.6^{ed}	64.8^{k}	11.6^{v}	16.4^{c}	19.0^{c}	12.9^{d}	22.1^{ef}	14.0^{cb}
		5	9.2^{dc}	37.0^{h}	18.9^{cd}	60.7^{ij}	0.0^{tu}	16.4^{c}	9.5^{b}	10.0^{cd}	16.2^{d}	6.4^{b}
		2.5	6.6^{bc}	14.8^{d}	18.9^{cd}	56.6^{g}	-42.0^{ji}	7.3^{b}	9.5^{b}	10.0^{cd}	10.3^{c}	9.9^{a}
		1.25	2.6^{ba}	14.8^{d}	13.2^{b}	52.4^{dce}	-44.9^{i}	7.3^{b}	9.5^{b}	7.1^{cd}	7.4^{bc}	2.8^{a}
		0.625	1.3^{a}	9.3^{c}	11.3^{b}	51.0^{dc}	-46.4^{hi}	3.6^{ba}	9.5^{b}	4.3^{b}	7.4^{bc}	2.8^{a}
		1% acetone	2.6^{a}	1.9^{a}	0.0^{a}	0.0^{a}	0.0^{tu}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Water	48	10	0.0^{n}	27.5^{kj}	14.7^{ij}	41.4^{o}	-59.2^{qp}	0.0^{e}	10.5^{ih}	0.0^{k}	19.4^{j}	**10.6^{j}**
		5	0.0^{n}	20.0^{ih}	14.7^{ij}	37.9^{on}	-55.1^{qr}	0.0^{e}	10.5^{ih}	0.0^{k}	11.1^{ji}	2.1^{i}
		2.5	0.0^{n}	15.0^{h}	14.7^{ij}	34.5^{mn}	-46.9^{trs}	0.0^{e}	10.5^{ih}	0.0^{k}	11.1^{ji}	2.1^{i}
		1.25	0.0^{n}	15.0^{h}	11.8^{ih}	34.5^{mn}	-46.9^{trs}	0.0^{e}	10.5^{ih}	0.0^{k}	11.1^{ji}	2.1^{i}
		0.625	0.0^{n}	**15.0^{h}**	**11.8^{ih}**	**34.5^{mn}**	-42.9^{tus}	0.0^{e}	5.3^{ih}	0.0^{k}	11.1^{ji}	2.1^{i}
		control	0.0^{n}	0.0^{gf}	0.0^{gf}	0.0^{ih}	0.0^{y}	0.0^{e}	0.0^{ih}	0.0^{k}	0.0^{l}	0.0^{ih}
	72	10	22.1^{kl}	17.4^{g}	14.3^{f}	52.9^{ml}	-79.7^{g}	13.0^{d}	21.7^{gh}	10.5^{hg}	7.3^{gf}	6.6^{ed}
		5	16.2^{ij}	13.0^{gf}	14.3^{f}	50.6^{l}	-64.4^{jlk}	13.0^{d}	13.0^{f}	10.5^{hg}	7.3^{gf}	6.6^{ed}
		2.5	16.2^{ij}	4.3^{ed}	4.8^{e}	50.6^{l}	-61.0^{jlk}	13.0^{d}	0.0^{ed}	10.5^{hg}	3.6^{ef}	6.6^{ed}
		1.25	16.2^{ij}	4.3^{ed}	4.8^{e}	50.6^{l}	-61.0^{jlk}	13.0^{d}	0.0^{ed}	10.5^{hg}	3.6^{ef}	3.3^{cd}
		0.625	13.2^{ih}	0.0^{d}	0.0^{ed}	50.6^{l}	-57.6^{mlk}	13.0^{d}	0.0^{ed}	3.5^{fe}	3.6^{ef}	0.0^{cb}
		control	0.0^{dce}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}	0.0^{c}	0.0^{ed}	0.0^{e}	0.0^{ed}	0.0^{cb}
	120	10	19.7^{gh}	7.4^{c}	22.6^{ed}	64.8^{k}	-84.1^{b}	16.4^{c}	19.0^{c}	18.6^{e}	17.6^{ed}	11.3^{b}
		5	11.8^{de}	5.6^{bc}	18.9^{cd}	62.8^{kj}	-81.2^{cb}	14.5^{c}	19.0^{c}	12.9^{d}	16.2^{d}	4.2^{a}
		2.5	9.2^{dc}	5.6^{bc}	13.2^{b}	55.9^{ih}	-58.0^{fe}	7.3^{b}	19.0^{c}	8.6^{cd}	2.9^{b}	1.4^{a}
		1.25	9.2^{dc}	5.6^{bc}	13.2^{b}	55.9^{gf}	-58.0^{fg}	7.3^{b}	17.5^{c}	8.6^{c}	2.9^{b}	1.4^{a}
		0.625	9.2^{dc}	5.6^{bc}	11.3^{b}	53.1^{de}	-34.8^{mlk}	5.5^{b}	17.5^{c}	7.1^{cb}	1.5^{a}	0.0^{a}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

Values in bold represent the MIC of plant extracts in mg/mL. Values within a column of treatment having the same superscript are not significantly different ($P < 0.05$) according to the LSD test. **WA:** *Wickerhamomyces anomalus,* **ZB1:** *Zygosaccharomyces bailii* CY0757, **ZB2:** *Zygosaccharomyces bailii* IGC4242, **RD:** *Rhodotorula dairenensis,* **YL:** *Yarrowia lipolytica,* **LE:** *Lodderomyces elongisporus*, **CL:** *Cryptococcus laurentii,* **CP:** *Candida parapsilosis,* **SC1:** *Saccharomyces cerevisiae* ATCC26602, **SC2:** *Saccharomyces cerevisiae* IGC3507.

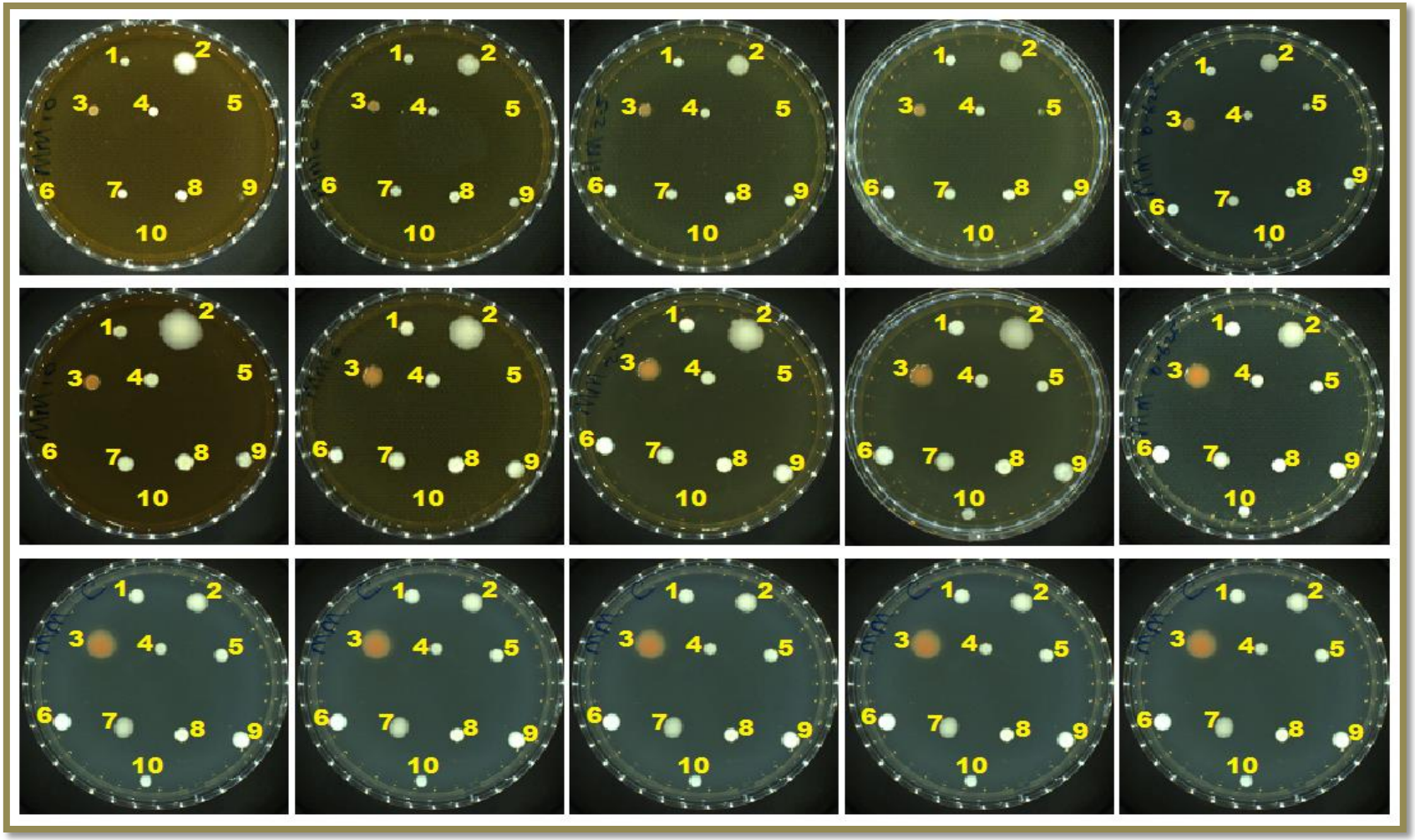

Figure 4.4. Methanol extract of *M. oleifera* showing growth of 1: *C. laurentii*, 2: *Y. lipolytica*, 3: *R. dairenensis*, 4*: L. elongisporus*, 5: *Z. bailii* CY0757, 6: *W. anomalus*, 7: *C. parapsilosis*, 8: *S. cerevisiae* ATCC26602, 9: *S. cerevisiae* IGC3507, 10: *Z. bailii* IGC4242 as concentration decreased from 10 - 0.625 mg/mL (left to right) after 48 hours (first row) and 120 hours (middle row) of incubation. Controls showing yeast growth after 120 hours of incubation (last row)

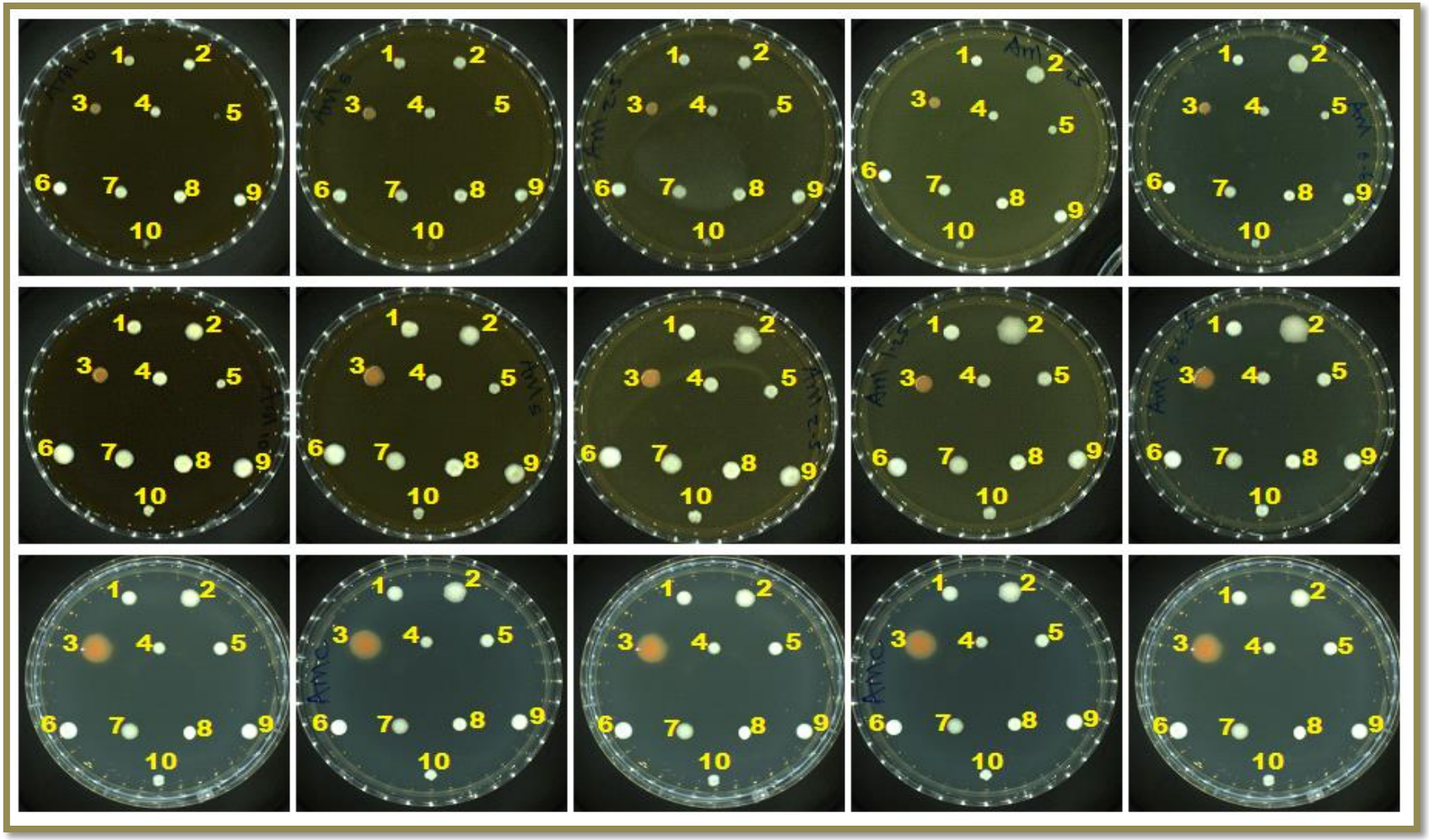

Figure 4.5. Acetone extract of *M. oleifera* showing growth of 1: *C. laurentii*, 2: *Y. lipolytica*, 3: *R. dairenensis*, 4: *L. elongisporus*, 5: *Z. bailii* CY0757, 6: *W. anomalus*, 7: *C. parapsilosis*, 8: *S. cerevisiae* ATCC26602, 9: *S. cerevisiae* IGC3507, 10: *Z. bailii* IGC4242 as concentration decreased from 10 - 0.625 mg/mL (left to right) after 48 hours (first row) and 120 hours (middle row) of incubation. Controls showing yeast growth after 120 hours of incubation (last row)

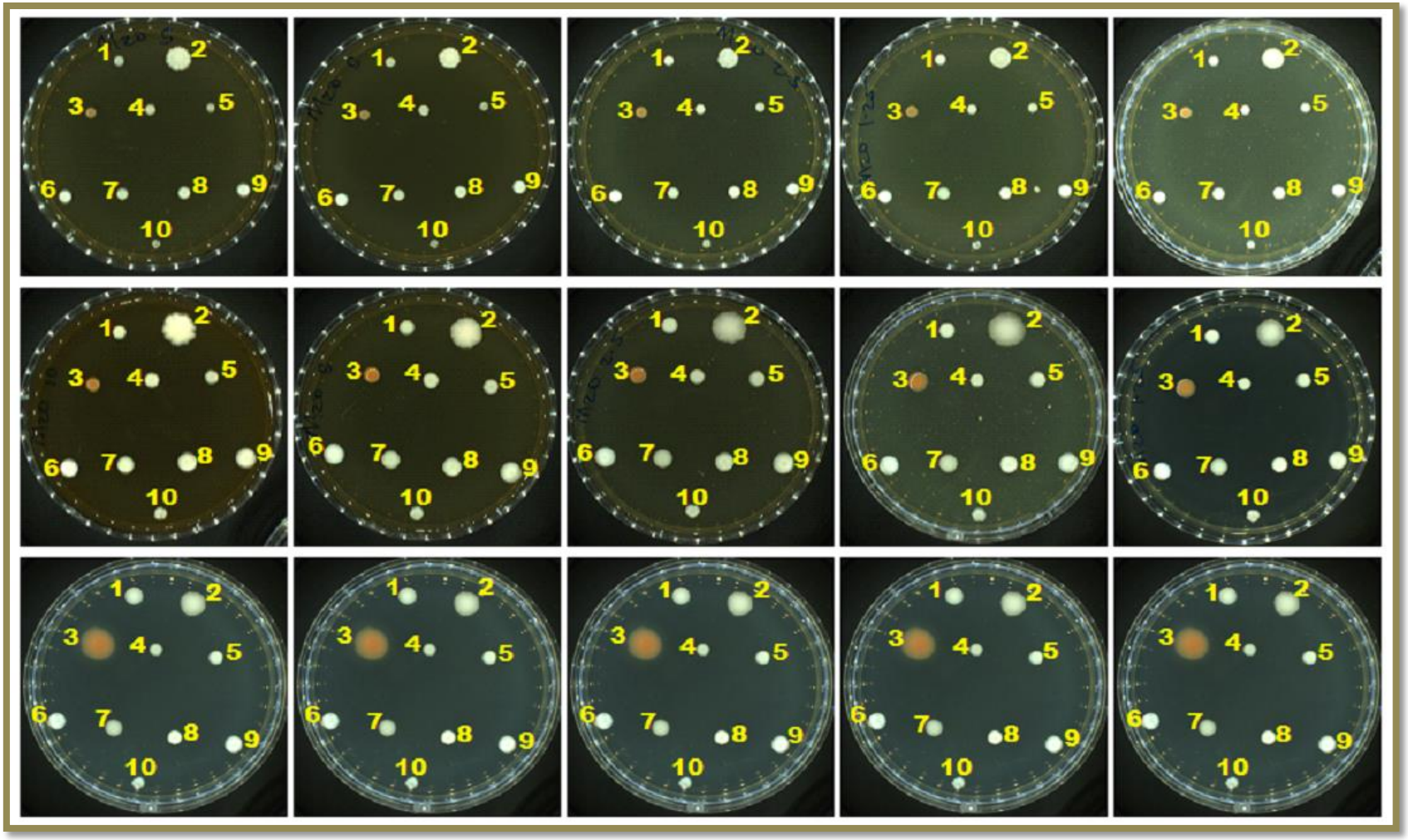

Figure 4.6. Water extract of *M. oleifera* showing growth of 1: *C. laurentii*, 2: *Y. lipolytica*, 3: *R. dairenensis*, 4: *L. elongisporus*, 5: *Z. bailii* CY0757, 6: *W. anomalus*, 7: *C. parapsilosis*, 8: *S. cerevisiae* ATCC26602, 9: *S. cerevisiae* IGC3507, 10: *Z. bailii* IGC4242 as concentration decreased from 10 - 0.625 mg/mL (left to right) after 48 hours (first row) and 120 hours (middle row) of incubation. Controls showing yeast growth after 120 hours of incubation (last row)

Gull et al. (2016) proposed that this may be attributed to the unstable nature of bioactive compounds in water. Notwithstanding, the possibility of some plant water extracts exhibiting antimicrobial activity is not entirely ruled out, for example, Gonelimali et al. (2018) demonstrated that the water extracts of roselle and clove had antimicrobial activity against some food pathogens and spoilage organisms. Another study by Pereira et al. (2007) confirmed that the water extracts of olive leaves showed a wide range of activity against Gram positive (*Staphylococcus aureus, Bacillus cereus* and *Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and fungi (*Cryptococcus neoformans* and *Candida albicans*). Gebreyohannes et al. (2019) also found that the hot water extract of wild mushrooms can inhibit growth of *Escherichia coli, Candida parapsilosis, Candida albicans, Staphylococcus aureus* and *Klebsiella pneumonia*.

The least affected yeasts by *X. aethiopica* after 120 hours were *C. parapsilosis, S. cerevisiae* ATCC26602 and IGC3507 with inhibition ranging from 0 - 29.8% (Table 4.2). The methanol extract (Figure 4.7) at a concentration of 1.25 mg/mL revealed 100% inhibition against the IGC4242 strain of *Z. bailii* and *C. laurentii* between 48 and 72 hours. However, after 120 hours, 35.8% inhibition was noted for *Z. bailii* IGC4242 at 2.5 mg/mL and 76.2% inhibition for *C. laurentii* at 5 mg/mL, indicating that the methanol extract was fungistatic against the yeasts at 1.25 mg/mL and fungicidal against the IGC4242 strain of *Z. bailii* at 2.5 mg/mL and *C. laurentii* at 5 mg/mL. Again the methanol extract at 10 mg/mL exhibited a fungistatic effect with 100% inhibition against *W. anomalus, Z. bailii* CY0757, *R. dairenensis* and *L. elongisporus* at 48 hours but as time increased to 120 hours, the inhibitory activity of the extract lessened with decreasing concentration.

Table 4.2. Growth inhibition of yeast (%) after treating with *X. aethiopica* extracts at different time intervals

Extract	Time (hours)	Concentration (mg/mL)	WA	ZB1	ZB2	RD	YL	LE	CL	CP	SC1	SC2
Methanol	48	10	100.0^{p}	100.0^{l}	100.0^{k}	100.0^{o}	44.9^{n}	100.0^{m}	100.0^{p}	15.0^{h}	5.6^{h}	27.7^{lk}
		5	42.5^{o}	42.5^{k}	100.0^{k}	60.3^{n}	44.9^{n}	**41.4^{l}**	100.0^{p}	15.0^{h}	5.6^{h}	27.7^{lk}
		2.5	37.5^{o}	32.5^{j}	100.0^{k}	50.0^{m}	40.8^{nm}	0.0^{j}	100.0^{p}	15.0^{h}	5.6^{h}	27.7^{lk}
		1.25	30.0^{lm}	32.5^{j}	100.0^{k}	50.0^{m}	40.8^{nm}	0.0^{j}	100.0^{p}	15.0^{h}	5.6^{h}	27.7^{lk}
		0.625	**27.5^{lm}**	**27.5^{ji}**	**100.0^{k}**	**44.8^{kl}**	**30.6^{l}**	0.0^{j}	**100.0^{p}**	**15.0^{h}**	5.6^{h}	**27.7^{lk}**
		1% methanol	0.0^{h}	5.0^{f}	0.0^{e}	1.7^{d}	2.0^{e}	0.0^{j}	2.6^{gh}	0.0^{g}	5.6^{h}	2.1^{fe}
		control	0.0^{h}	0.0^{fe}	0.0^{e}	0.0^{dc}	0.0^{e}	0.0^{j}	0.0^{gh}	0.0^{g}	0.0^{h}	0.0^{e}
	72	10	63.2^{o}	37.0^{jl}	100.0^{k}	70.6^{n}	50.8^{nm}	45.7^{k}	100.0^{p}	29.8^{g}	34.5^{h}	34.4^{hı}
		5	57.4^{lm}	37.0^{ji}	100.0^{k}	65.9^{m}	50.8^{nm}	37.0^{j}	100.0^{p}	29.8^{g}	27.3^{g}	34.4^{hi}
		2.5	57.4^{lk}	34.8^{hı}	100.0^{k}	57.6^{j}	42.4^{l}	30.4^{hg}i	100.0^{p}	29.8^{g}	27.3^{g}	31.1^{hg}
		1.25	55.9^{lk}	34.8^{hı}	100.0^{k}	57.6^{j}	39.0^{lk}	26.1^{g}	100.0^{p}	29.8^{g}	27.3^{g}	27.9^{fg}
		0.625	52.9^{i}	30.4^{hg}	35.7^{i}	52.9^{ih}	32.2^{ji}	26.1^{g}	34.1^{l}	22.8^{f}	23.6^{g}	24.6^{fe}
		1% methanol	1.5^{b}	0.0^{b}	0.0^{c}	1.2^{b}	1.7^{c}	0.0^{e}	0.0^{d}	0.0^{b}	3.6^{e}	3.3^{c}
		control	0.0^{b}	0.0^{b}	0.0^{c}	0.0^{b}	0.0^{c}	0.0^{e}	0.0^{ed}	0.0^{b}	0.0^{e}	0.0^{cb}
	120	10	46.1^{hg}	20.8^{cde}	100.0^{k}	80.0^{m}	47.8^{lk}	41.8^{hgi}	100.0^{p}	25.7^{d}	20.6^{de}	22.5^{d}
		5	43.4^{g}	18.9^{cd}	100.0^{k}	74.5^{lj}	47.8^{lk}	40.0^{hg}	100.0^{p}	25.7^{d}	20.6^{de}	16.9^{c}
		2.5	43.4^{g}	18.9^{cd}	100.0^{k}	66.9^{g}	37.7^{gfh}	21.8^{f}	76.2^{o}	25.7^{d}	20.6^{de}	16.9^{c}
		1.25	43.4^{g}	17.0^{cb}	35.8^{e}	64.8^{g}	37.7^{gfh}	21.8^{f}	54.0^{l}	25.7^{d}	20.6^{de}	11.3^{b}
		0.625	39.5^{f}	13.2^{b}	35.8^{e}	60.7^{d}	30.4^{e}	21.8^{f}	36.5^{gf}	18.6^{b}	16.2^{cb}	11.3^{b}
		1% methanol	1.3^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{ba}	3.2^{a}	0.0^{a}	0.0^{a}	0.0^{a}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Acetone	48	10	42.5^{o}	42.5^{k}	100.0^{k}	60.3^{n}	61.2^{o}	**20.7^{k}**	100.0^{p}	25.0^{i}	16.7^{i}	31.9^{l}
		5	32.5^{nm}	42.5^{k}	100.0^{k}	50.0^{m}	40.8^{nm}	0.0^{j}	100.0^{p}	15.0^{h}	5.6^{h}	27.7^{lk}
		2.5	27.5^{lm}	32.5^{j}	100.0^{k}	50.0^{m}	40.8^{nm}	0.0^{j}	44.7^{n}	15.0^{h}	5.6^{h}	27.7^{lk}
		1.25	25.0^{lk}	27.5^{ji}	100.0^{k}	41.4^{kj}	30.6^{l}	0.0^{j}	34.2^{m}	15.0^{h}	5.6^{h}	27.7^{lk}
		0.625	**25.0^{lk}**	**15.0^{g}**	**17.6^{hi}**	**41.4^{kj}**	**22.4^{jk}**	0.0^{j}	**23.7^{l}**	**15.0^{h}**	5.6^{h}	**0.0^{ji}**
		1% acetone	0.0^{h}	5.0^{f}	5.9^{fe}	1.7^{d}	2.0^{e}	0.0^{j}	0.0^{gh}	0.0^{g}	0.0^{h}	0.0^{e}
		control	0.0^{h}	0.0^{fe}	0.0^{e}	0.0^{d}	0.0^{e}	0.0^{j}	0.0^{gh}	0.0^{g}	0.0^{h}	0.0^{e}
	72	10	57.4^{lm}	37.0^{jl}	100.0^{k}	65.9^{m}	54.2^{n}	34.8^{hjı}	100.0^{p}	29.8^{g}	34.5^{h}	31.1^{hg}
		5	50.0^{ji}	34.8^{hi}	100.0^{k}	62.4^{kl}	50.8^{nm}	34.8^{hji}	47.7^{nm}	29.8^{g}	27.3^{g}	27.9^{fg}
		2.5	50.0^{ji}	30.4^{hg}	31.0^{hg}	52.9^{ih}	32.2^{ji}	26.1^{g}	22.7^{kj}	22.8^{f}	16.4^{f}	24.6^{fe}
		1.25	50.0^{ji}	26.1^{g}	31.0^{hg}	50.6^{h}	32.2^{ji}	26.1^{g}	22.7^{kj}	22.8^{f}	16.4^{f}	24.6^{fe}
		0.625	41.2^{h}	26.1^{g}	28.6^{fg}	50.6^{h}	32.2^{ji}	26.1^{g}	18.2^{ihj}	19.3^{e}	0.0^{f}	0.0^{fe}
		1% acetone	1.5^{b}	0.0^{b}	0.0^{c}	1.2^{b}	1.7^{c}	0.0^{de}	0.0^{d}	0.0^{b}	0.0^{cde}	0.0^{cb}
		control	0.0^{b}	0.0^{b}	0.0^{c}	0.0^{b}	0.0^{cb}	0.0^{e}	0.0^{ed}	0.0^{b}	0.0^{cde}	0.0^{cb}

	120	10	39.5^{t}	24.1^{de}	73.6^{l}	76.6^{kj}	58.0^{nm}	25.5^{t}	77.8^{o}	27.1^{d}	20.6^{de}	21.1^{d}
		5	39.5^{f}	22.2^{cde}	35.8^{e}	71.0^{h}	42.0^{ji}	21.8^{f}	44.4^{ij}	25.7^{d}	17.6^{cd}	21.1^{d}
		2.5	39.5^{f}	22.2^{cde}	35.8^{e}	64.1^{f}	34.8^{f}	16.4^{e}	33.3^{ef}	25.7^{d}	17.6^{cd}	16.9^{c}
		1.25	39.5^{f}	20.4^{cd}	35.8^{e}	61.4^{de}	29.0^{e}	12.7^{de}	33.3^{ef}	22.9^{c}	16.2^{cb}	14.1^{cb}
		0.625	32.9^{e}	14.8^{b}	24.5^{d}	57.9^{c}	23.2^{d}	12.7^{de}	27.0^{d}	20.0^{b}	0.0^{b}	0.0^{b}
		1% acetone	2.6^{a}	1.9^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{ba}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{ba}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
Water	48	10	27.5^{lm}	20.0^{hg}	14.7^{hg}	50.0^{m}	28.6l	0.0^{j}	44.7^{n}	**15.0^{h}**	5.6^{h}	27.7^{lk}
		5	25.0^{lk}	15.0^{hg}	14.7^{hg}	50.0^{m}	22.4^{jk}	0.0^{j}	34.2^{m}	0.0^{g}	5.6^{h}	23.4^{jk}
		2.5	15.0^{ji}	**5.0^{g}**	**14.7^{hg}**	41.4kj	22.4^{jk}	0.0^{j}	23.7^{l}	0.0^{g}	5.6^{h}	14.9^{hi}
		1.25	15.0^{ji}	0.0^{f}	5.9^{fe}	41.4^{kj}	16.3^{ih}	0.0^{j}	**15.8^{k}**	0.0^{g}	5.6^{h}	14.9^{hi}
		0.625	15.0^{ji}	0.0^{fe}	0.0^{fe}	**41.4^{kj}**	**12.2^{gfh}**	0.0^{j}	5.3^{ihj}	0.0^{g}	0.0^{h}	**10.6^{hg}**
		control	0.0^{h}	0.0^{fe}	0.0^{fe}	0.0^{dc}	0.0^{e}	0.0^{l}	0.0^{gh}	0.0^{g}	0.0^{h}	0.0^{e}
		10	41.2^{h}	34.8^{hi}	14.7^{hg}	60.0^{kj}	49.2^{m}	26.1^{g}	22.7^{kj}	29.8^{g}	16.4^{f}	24.6^{fe}
	72	5	41.2^{h}	26.1^{g}	14.7^{hg}	60.0^{kj}	42.4^{l}	26.1^{g}	22.7^{kj}	22.8^{f}	16.4^{f}	24.6^{fe}
		2.5	40.9^{h}	26.1^{g}	14.7^{hg}	50.6^{h}	35.6^{jk}	26.1^{g}	18.2^{ihj}	19.3^{e}	16.4^{f}	24.6^{fe}
		1.25	40.9^{h}	26.1^{g}	0.0^{e}	50.6^{h}	28.8^{gih}	26.1^{g}	18.2^{ihj}	19.3^{e}	16.4^{f}	24.6^{fe}
		0.625	40.9^{h}	26.1^{g}	0.0^{e}	50.6^{h}	25.4^{gf}	26.1^{g}	9.1^{gf}	19.3^{e}	16.4^{f}	24.6^{fe}
		control	0.0^{b}	0.0^{b}	0.0^{e}	0.0^{b}	0.0^{cb}	0.0^{e}	0.0^{ed}	0.0^{b}	0.0^{cde}	0.0^{cb}
		10	31.6^{ed}	13.2^{b}	13.2^{b}	71.0^{h}	17.4^{c}	10.9^{dc}	19.0^{c}	27.1^{d}	16.2^{cb}	11.3^{b}
	120	5	28.9^{d}	13.2^{b}	13.2^{b}	66.9^{g}	11.6^{b}	10.9^{dc}	19.0^{c}	25.7^{d}	16.2^{cb}	11.3^{b}
		2.5	25.0^{c}	13.2^{b}	13.2^{b}	63.4^{g}	0.0^{b}	3.6^{bc}	9.5b	18.6^{c}	13.2^{cb}	11.3^{b}
		1.25	25.0^{c}	13.2^{b}	13.2^{b}	63.4^{fe}	0.0^{a}	3.6^{ba}	9.5^{b}	18.6^{c}	13.2^{b}	11.3^{b}
		0.625	25.0^{c}	13.2^{b}	13.2^{b}	57.9^{c}	0.0^{a}	0.0^{a}	9.5^{b}	18.6^{b}	13.2^{b}	0.0^{b}
		control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

Values in bold represent the MIC of plant extracts in mg/mL. Values within a column of treatment having the same superscript are not significantly different (P < 0.05) according to the LSD test. **WA:** *Wickerhamomyces anomalus,* **ZB1:** *Zygosaccharomyces bailii* CY0757, **ZB2:** *Zygosaccharomyces bailii* IGC4242, **RD:** *Rhodotorula dairenensis,* **YL:** *Yarrowia lipolytica,* **LE:** *Lodderomyces elongisporus*, **CL:** *Cryptococcus laurentii,* **CP:** *Candida parapsilosis,* **SC1:** *Saccharomyces cerevisiae* ATCC26602, **SC2:** *Saccharomyces cerevisiae* IGC3507

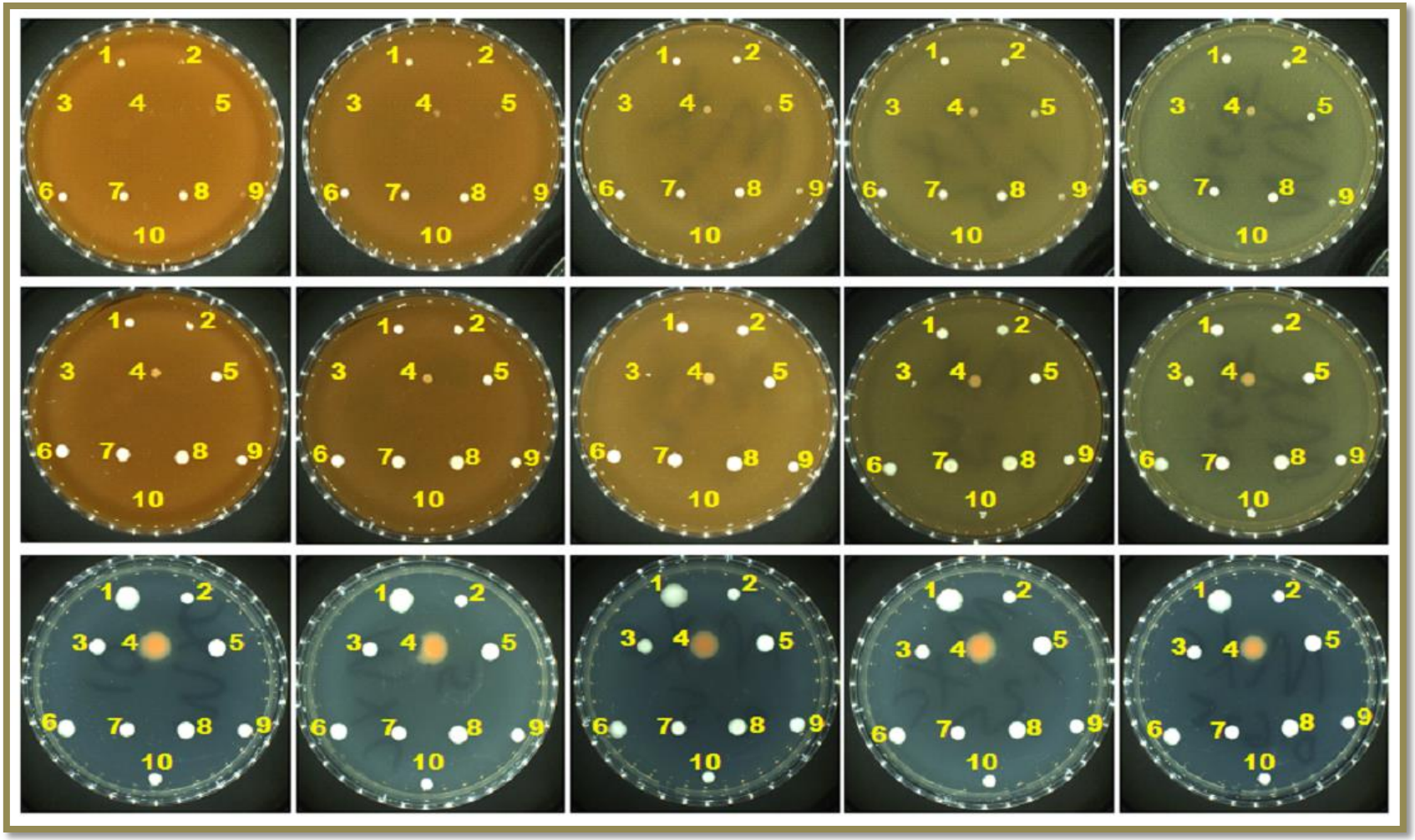

Figure 4.7. Methanol extract of X. aethiopica showing growth of 1: *Y. lipolytica*, 2: *L. elongisporus*, 3: *C. laurentii*, 4: *R. dairenensis*, 5: *W. anomalus*, 6: *C. parapsilosis*, 7: *S. cerevisiae* ATCC26602, 8: *S. cerevisiae* IGC3507, 9: *Z. bailii* CY0757, 10: *Z. bailii* IGC4242 as concentration decreased from 10 - 0.625 mg/mL (left to right) after 48 hours (first row) and 120 hours (middle row) of incubation. Controls showing yeast growth after 120 hours of incubation (last row)

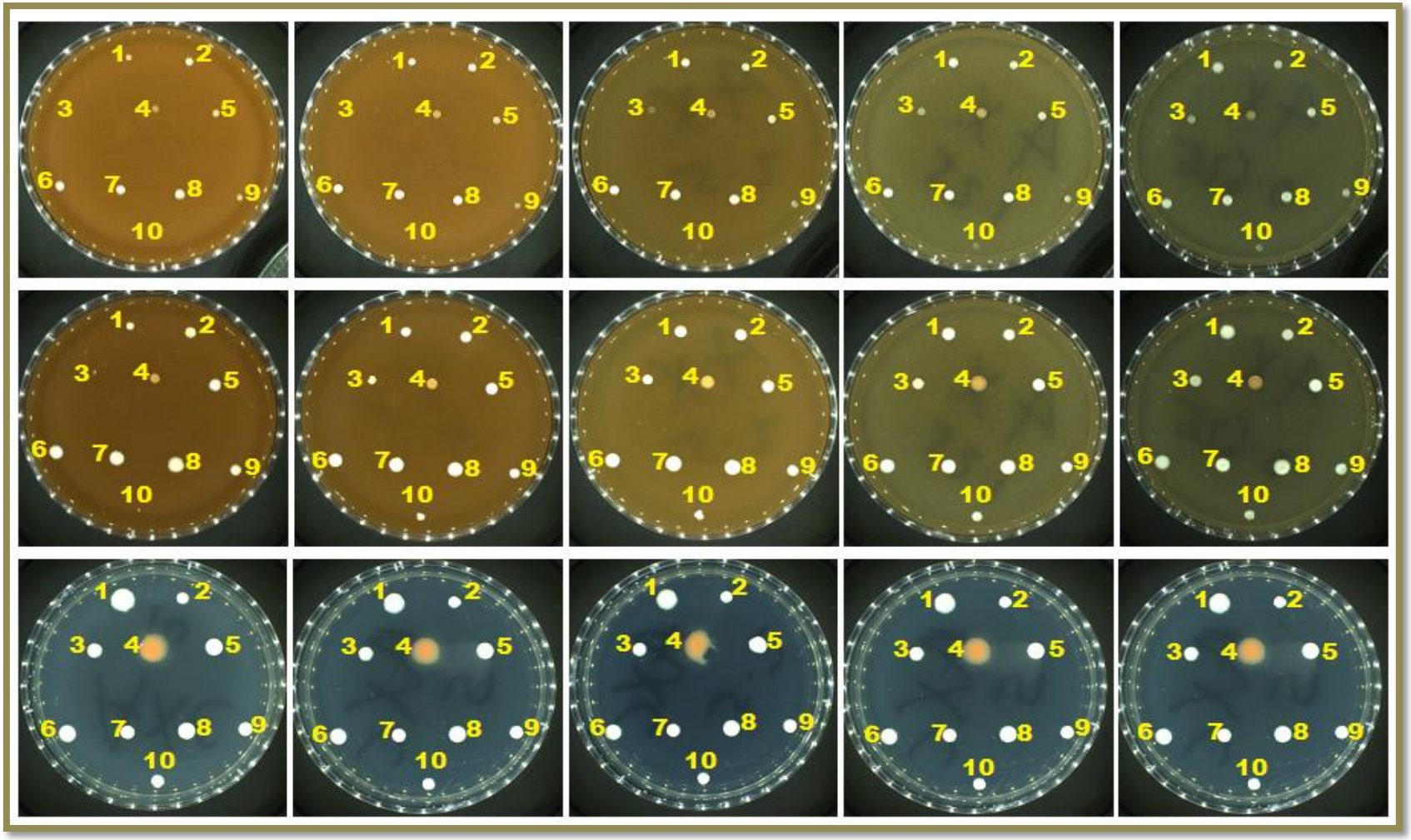

Figure 4.8. Acetone extract of *X. aethiopica* showing growth of 1: *Y. lipolytica*, 2: *L. elongisporus*, 3: *C. laurentii*, 4: *R. dairenensis*, 5: *W. anomalus*, 6: *C. parapsilosis*, 7: *S. cerevisiae* ATCC26602, 8: *S. cerevisiae* IGC3507, 9: *Z. bailii* CY0757, 10: *Z. bailii* IGC4242 as concentration decreased from 10 - 0.625 mg/mL (left to right) after 48 hours (first row) and 120 hours (middle row) of incubation. Controls showing yeast growth after 120 hours of incubation (last row)

Although the acetone extract of *X. aethiopica* (Figure 4.8) did not show fungicidal activity against any of the tested yeasts after 120 hours, it exerted 100% inhibition against *Z. bailii* IGC4242 at 1.25 and 5 mg/mL and against *C. laurentii* at 5 and 10 mg/mL after 48 and 72 hours respectively. The acetone extract also showed inhibitory activity ranging from 41.4 - 76.6% for *R. dairenensis,* 22.4 - 61.2% for *Y. lipolytica* and 25.0 - 57.4% for W. *anomalus* which was considerable compared to *L. elongisporus* and *Z. bailii* CY0757 that showed less than 50% inhibitory activity across the times. Again, these results were unexpected as Dzoyem et al. (2016) reported that antimicrobial compounds from plants are more soluble in acetone. This confirms that 50% concentration of acetone used for extraction in this study, affected the solubility of anti-yeast compounds, thereby reducing the performance of the acetone extract. Nonetheless, the acetone extract of *X. aethiopica* may be used as a fungistatic agent for *C. laurentii* and the IGC4242 strain of *Z. bailii.*

The water extract of *X. aethiopica* corresponded to the water extract of *M. oleifera* in that it displayed the least bioactivity against the yeasts and exhibited up to 50% inhibition against *R. dairenensis* after 72 and 120 hours at 0.625 mg/mL.

Studies have reported the antifungal and antibacterial activities of *X. aethiopica* against the growth of some spoilage and pathogenic organisms in food. For example, Sokamte et al. (2018) demonstrated that essential oil from the fruits of *X. aethiopica* is effective in the control of *Aspergillus niger* and *Fusarium oxysporum*. Ogbonna et al. (2013) also showed that *X. aethiopica* fruit extract can inhibit the growth of *Saccharomyces cerevisiae, Penicillium funiculosum, Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureaus.* Hence, in this study it was envisaged that extracts from this plant would show some level of antimicrobial activity.

In addition to the antimicrobial activity shown in this study, high antioxidant activity of *X. aethiopica* extracts has been demonstrated in Chapter three. These results therefore indicate that *X. aethiopica* is not just a spice that could enhance the flavour of foods but its consumption can boost the body's immunity to diseases by raising

the antioxidant levels in the body. The use of *X. aethiopica* in traditional medicine for preventing and curing diseases is thus validated (Ngwoke et al., 2015; Adefegha et al., 2018).

As depicted in Table 4.1 and 4.2, growth inhibition (%) within a column of treatment were significantly different across the time intervals (48, 72 and 120 hours), signifying that the length of time the yeasts were exposed to the extracts had an influence on the level of anti-yeast or stimulatory activity. Also, there were significant differences between various concentrations of extracts used particularly against *Y. lipolytica, R. dairenensis, W. anomalus, Z. bailii* CY0757 and IGC4242 for *M. oleifera* and *Z. bailii* CY0757 and IGC4242, *C. laurentii, R. dairenensis, W. anomalus, L. elongisporus* and *Y. lipolytica* for *X. aethiopica*. It was observed that the anti-yeast and stimulatory activity of the plant extracts rose as concentration increased to 10 mg/mL.

It is known that secondary metabolites from plant sources have broad spectrum activity against microorganisms (Hugo and Hugo, 2015; Kone et al., 2019). In that light, the variation observed in the yeasts sensitivity to the extracts may be ascribed to the presence of bioactive compounds in different concentrations. The solvents used for extraction also may have played a role since the polarity of solvents is mainly accountable for the amount of active compounds extracted from plants (Wanyo et al., 2016; Tohma et al., 2019). Moreover, the solubility of the extracted anti-yeast compounds in 50% methanol may justify the highest activity exhibited by the methanol extract of both plants (Table 4.1 and 4.2). This makes 50% methanol the best solvent for extracting anti-yeast compounds from *M. oleifera* leaves and *X. aethiopica* fruits.

4.4.2. Microbiological analysis of treated fruit juices

Figure 4.9 represents the microbial analysis of the untreated grape and orange juice which served as controls. It is evident from the figure that the fruit juices were contaminated prior to inoculation with the target organisms. This contamination may

have been caused by unhygienic conditions or practices during handling and processing of fruits. This highlights the importance of aseptic practices in the fruit juice industry as they help to minimise the risks of contamination and extends the shelf life of the fruit juices. Another possible source of contamination might be the inadequate washing of the fruits before juicing, taking into account that microbes constitute the natural flora of raw fruits (Oluwole et al., 2016). It is thus necessary to wash raw fruits with clean sterile water and traditional food sanitisers such as chlorine before juicing in order to avoid cross-contamination.

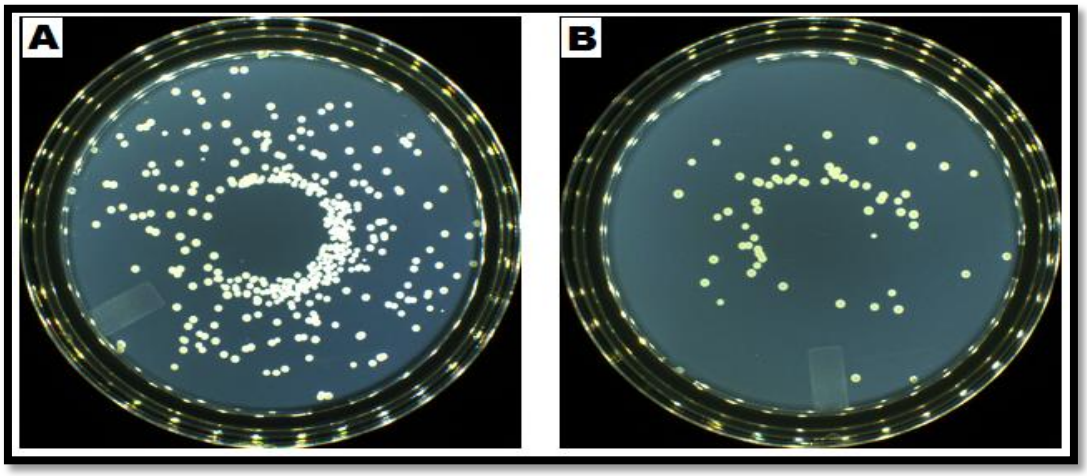

Figure 4.9. (A) Untreated grape juice prior to inoculation with spoilage yeasts (B) Untreated orange juice prior to inoculation with spoilage yeast

The microbiological analysis for the treated fruit juices after 3 days (Figure 4.10) revealed growth that was innumerable. Owing to the presence of contaminants in the controls (Figure 4.9), it is erroneous to conclude that the plant extracts did not inhibit the growth of the yeasts inoculated into the fruit juices. This is why the inclusion of controls in scientific experiments is important as they form a basis for comparison.

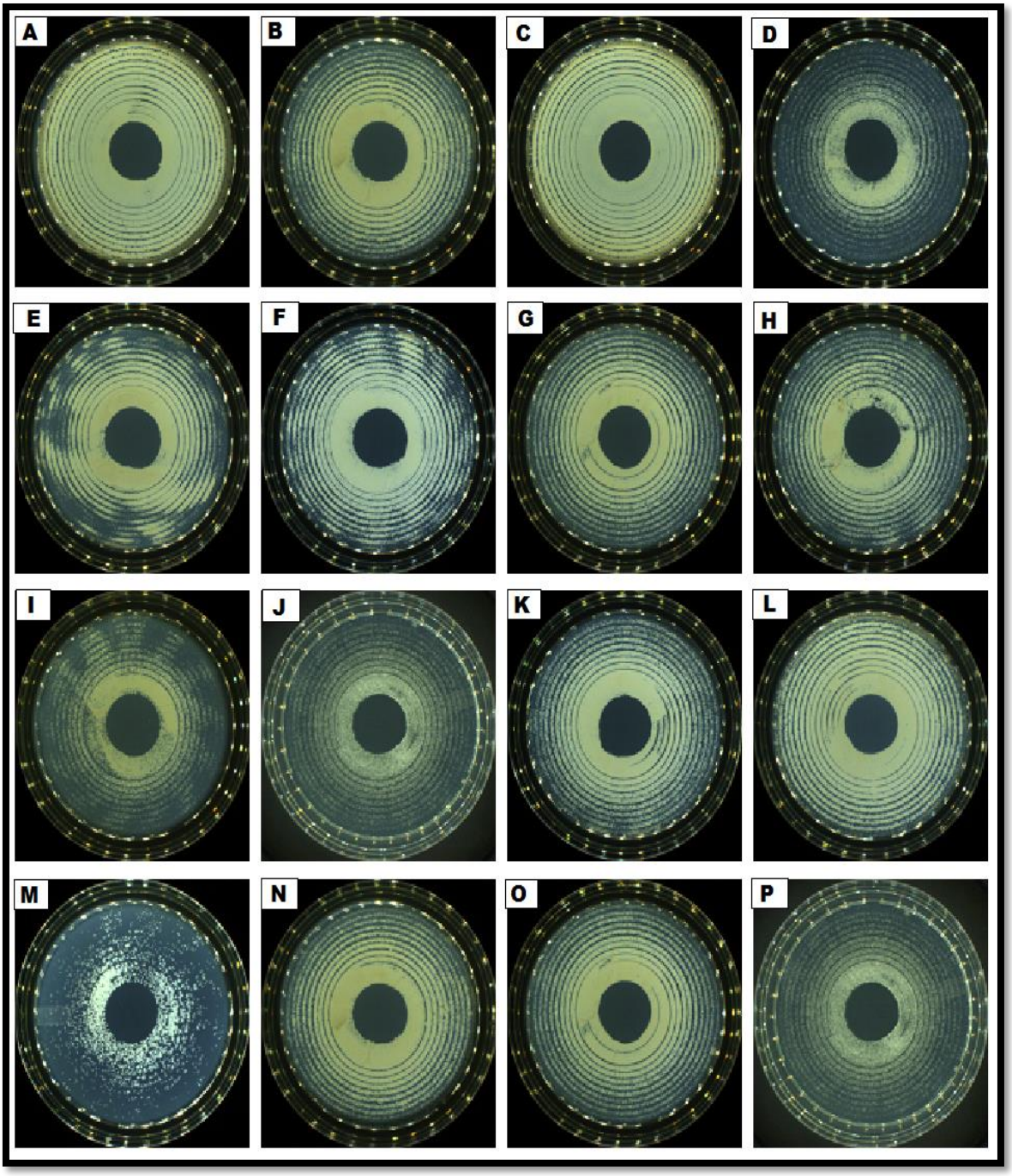

Figure 4.10. *M. oleifera* orange juice containing *Z. bailii* CY0757 (A,B) and *Z. bailii* IGC4242 (C,D) at 10 and 5 mg/mL respectively, *M. oleifera* grape juice containing *Z. bailii* CY0757 (E,F) and *Z. bailii* IGC4242 (G,H) at 10 and 5 mg/mL respectively, *X. aethiopica* orange juice containing *Z. bailii* IGC4242 (I,J) and *C. laurentii* (K,L) at 10 and 5 mg/mL respectively, *X. aethiopica* grape juice containing *Z. bailii* IGC4242 (M,N) and *C. laurentii* (O,P) at 10 and 5 mg/mL respectively.

4.5. Conclusion

This study has demonstrated that *M. oleifera* and *X. aethiopica* extracts can inhibit the growth of some yeasts involved in fruit juice spoilage *in vitro*. However, anti-yeast activity of plant extracts in fresh grape and orange juice was inconclusive. Microbiological analysis of fruit juices used as controls showed that the juices had been contaminated prior to inoculating with the target yeasts. The microflora of the raw fruits may have been introduced into the fruit juices in the course of handling and processing. It is noteworthy to consider pre-washing the fruits with chlorine-based sanitizer and practice good hygiene during handling and processing to reduce the risks of contaminations in future experiments.

In conclusion, *M. oleifera* and *X. aethiopica* have limited potential for use as natural antimicrobial preservatives in fruit juice due to their inability to inhibit the growth of certain fruit juice spoilage yeasts in this study.

4.6. References

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Almeida, E.T.C., de Souza, G.T., Guedes, J.P.S., Barbosa, I.M., de Sousa, C.P., Castellano, L.R.C., Magnani, M. and de Souza, E.L. 2019. *Mentha piperita* L. essential oil inactivates spoilage yeasts in fruit juices through the perturbation of different physiological functions in yeast cells. *Food Microbiology,* 82: 20-29.

Aneja, K.R., Dhiman, R., Aggarwal, N.K., Kumar, V. and Kaur, M. 2014. Microbes associated with freshly prepared juices of citrus and carrots: A review. *International Journal of Food Science*, 2014: 1-7.

Bevilacqua, A., Corbo, M.R., Campaniello, D., D'Amato, D., Gallo, M., Speranza, B. and Sinigaglia, M. 2011. Shelf life prolongation of fruit juices through essential

oils and homogenization: A review. Science against microbial pathogens: Communicating current research and technological advances. *Microbiology,* 3: 1157-1166.

Biango-Daniels, M.N., Snyder, A.B., Worobo, R.W. and Hodge, K.T. 2019. Fruit infected with *Paecilomyces niveus*: A source of spoilage inoculum and patulin in apple juice concentrate? *Food Control,* 97: 81-86.

Bukvicki, D., Gottardi, D., Tyagi, A.K., Veljic, M., Marin, P.D., Vujisic, L., Guerzoni, M.E. and Vannini, L. 2014. *Scapania nemorea* liverwort extracts: Investigation on volatile compounds, *in vitro* antimicrobial activity and control of *Saccharomyces cerevisiae* in fruit juice. *LWT-Food Science and Technology,* 55: 452-458.

Cardador, M.J. and Gallego, M. 2015. Haloacetic acids content of fruit juices and soft drinks. *Food Chemistry,* 173: 685-693.

Corbett, K.M. and de Smidt, O. 2019. Culture-dependent diversity profiling of spoilage yeasts species by PCR-RFLP comparative analysis. *Food Science and Technology International,* 25: 671-679.

Darvishi, F., Fathi, Z., Ariana, M. and Moradi, H. 2017. *Yarrowia lipolytica* as a workhorse for biofuel production. *Biochemical Engineering Journal,* 127: 87-96.

Dzoyem, J.P., Aro, A.O., McGaw, L.J. and Eloff, J.N. 2016. Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in southern Africa to treat tuberculosis and respiratory ailments. *South African Journal of Botany,* 102: 70-74.

Gebreyohannes, G., Nyerere, A., Bii, C. and Sbhatu, D.B. 2019. Determination of antimicrobial activity of extracts of indigenous wild mushrooms against pathogenic organisms. *Evidence-Based Complementary and Alternative Medicine*, 2019: 1-7.

Gonçalves, F.A.G., Colen, G. and Takahashi, J.A. 2014. *Yarrowia lipolytica* and its multiple applications in the biotechnological industry. *The Scientific World Journal*, 2014: 1-14.

Gonelimali, F.D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M. and Hatab, S.R. 2018. Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. *Frontiers in Microbiology,* 9: 1639.

Guedes, J.P.S., da Costa Medeiros, J.A., de Souza e Silva, R.S., de Sousa, J.M.B., da Conceição, M.L. and de Souza, E.L. 2016. The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices. *International Journal of Food Microbiology,* 238: 183-192.

Gull, I., Javed, A., Aslam, M.S., Mushtaq, R. and Athar, M.A. 2016. Use of *Moringa oleifera* flower pod extract as natural preservative and development of SCAR marker for its DNA based identification. *BioMed Research International,* 2016: 1-12.

Hernández, A., Pérez-Nevado, F., Ruiz-Moyano, S., Serradilla, M.J., Villalobos, M.C., Martín, A. and Córdoba, M.G. 2018. Spoilage yeasts: What are the sources of contamination of foods and beverages? *International Journal of Food Microbiology,* 286: 98-110.

Hugo, C.J. and Hugo, A. 2015. Current trends in natural preservatives for fresh sausage products. *Trends in Food Science and Technology,* 45: 12-23.

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S. and Weththasinghe, P. 2015. Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. *LWT - Food Science and Technology*, 64: 1204-1208.

Kone, A.P., Desjardins, Y., Gosselin, A., Cinq-Mars, D., Guay, F. and Saucier, L. 2019. Plant extracts and essential oil product as feed additives to control rabbit meat microbial quality. *Meat Science,* 150: 111-121.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

López-Carballo, G., Gómez-Estaca, J., Catalá, R., Hernández-Muñoz, P. and Gavara, R. 2012. Active antimicrobial food and beverage packaging. In:

Emerging Food Packaging Technologies: Principles and Practice. Yam, K.L. and Lee, D.S., Eds. València, Spain: Woodhead Publishing Series in Food Science, Technology and Nutrition, pp. 27-54.

Maciel, N.O.P., Piló, F.B., Freitas, L.F.D., Gomes, F.C.O., Johann, S., Nardi, R.M.D., Lachance, M.A. and Rosa, C.A. 2013. The diversity and antifungal susceptibility of the yeasts isolated from coconut water and reconstituted fruit juices in Brazil. *International Journal of Food Microbiology,* 160: 201-205.

Ng, K.R., Lyu, X., Mark, R. and Chen, W.N. 2019. Antimicrobial and antioxidant activities of phenolic metabolites from flavonoid-producing yeast: Potential as natural food preservatives. *Food Chemistry,* 270: 123-129.

Ngwoke, K.G., Ikeanyi, A.U., Eze, P.M., Ezemokwe, I.C., Abba, C.C. and Ugwu, M.C. 2015. Phytochemical and antioxidant properties of extracts of *Xylopia aethiopica* fruits. *Chemical Science Review and Letters*, 4(13): 267-270.

Ogbonna C.N., Nozaki, K. and Yajima, H. 2013. Antimicrobial activity of *Xylopia aethiopica*, *Aframomum melegueta* and *Piper guineense* ethanolic extracts and the potential of using *Xylopia aethiopica* to preserve fresh orange juice. *African Journal of Biotechnology,* 12(16): 1993-1998.

Oluwole, O.A., David, O.M., Falegan, C.R., Awojuyigbe, B. and Olajide, O.M. 2016. Microbiological and physicochemical properties of commercial seal tampered refrigerated fruit juices. *International Journal of Biological Research,* 4(1): 21-24.

Pereira, A.P., Ferreira, I.C., Marcelino, F., Valentão, P., Andrade, P.B., Seabra, R., Estevinho, L., Bento., A. and Pereira, J.A. 2007. Phenolic compounds and antimicrobial activity of olive (*Olea europaea L.* Cv. Cobrançosa) leaves. *Molecules, 12*(5): 1153-1162.

Snyder, A.B. and Worobo, R.W. 2018. The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers. *Food Control,* 85: 144-150.

Sokamte, T.A., Beumo, T.M.N., Dongmo, P.M.J. and Ngoune, L.T. 2018. Essential oil of *Xylopia aethiopica* from Cameroon: Chemical composition, antiradical

and *in vitro* antifungal activity against some mycotoxigenic fungi. *Journal of King Saud University-Science,* 30: 466-471.

Soto, K.M., Iturriaga, M.H., Piña, G.L., Bárcenas, G.L. and Mendoza, S. 2019. Antimicrobial effect of nisin electrospun amaranth: Pullulan nanofibers in apple juice and fresh cheese. *International Journal of Food Microbiology,* 295: 25-32.

Tohma, H., Altay, A., Köksal, E., Gören, A.C. and Gülçin, I. 2019. Measurement of anticancer, antidiabetic and anticholinergic properties of sumac (*Rhus coriaria*): Analysis of its phenolic compounds by LC–MS/MS. *Journal of Food Measurement and Characterization,* 13(2): 1607-1619.

Wang, H., Hu, Z., Long, F., Guo, C., Niu, C., Yuan, Y. and Yue, T. 2016. Combined effect of sugar content and pH on the growth of a wild strain of *Zygosaccharomyces rouxii* and time for spoilage in concentrated apple juice. *Food Control,* 59: 298-305.

Wanyo, P., Kaewseejan, N., Meeso, N. and Siriamornpun, S. 2016. Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by-products. *Applied Biological Chemistry,* 59(3): 373-384.

Chapter Five

In vitro cytotoxicity of *Moringa oleifera* and *Xylopia aethiopica* extracts

5.1. Abstract

Plant secondary metabolites have recently gained significant interest to the food and beverage, pharmaceutical, cosmetics and pesticides industries owing to the rise in demand for natural products. However, some plants are potentially toxic to humans and it is imperative to be aware of such plants and the compound(s) responsible for their toxicity. The aim of this study was to investigate the *in vitro* cytotoxicity of methanol, acetone and water extracts of *Moringa oleifera* leaves and *Xylopia aethiopica* fruits. Vero cells were treated with the plant extracts at 100, 200 and 400 µg/mL for 48 hours using the Hoechst 33342 and propidium iodide (PI) dual staining procedure. The results showed that *M. oleifera* extracts were not cytotoxic to the cells at all concentrations. Although the methanol and acetone extracts of *X. aethiopica* revealed toxicity at all concentrations, the water extract did not show toxicity even at the highest concentration of 400 µg/mL. This study has demonstrated that all the tested extracts except the methanol and acetone extract of *X. aethiopica* were not toxic on normal Vero cells. Therefore, incorporating them into fruit juices as natural preservatives may not be detrimental to the human health.

Keywords: Plant secondary metabolites, cytotoxicity, Vero cell lines, cell viability, fruit juice preservative.

5.2. Introduction

Plants have served as good sources of food and medicine to humans and animals from time immemorial (Moosavi et al., 2018). Nowadays, different industries are bestowing more attention to plants and plant-derived compounds due to consumers' inclination towards natural products. The medical and pharmaceutical industries, for instance, use active compounds from plants to formulate safer and effective novel drugs (Soliman et al., 2017). Likewise, producers of cosmetics incorporate plant extracts and essential oils into their formulations as safer, hypo-allergenic alternatives to BHT (Butylated Hydroxytoluene) and BHA (Butylated Hydroxyanisole) which are traditionally used to preserve cosmetics, drugs, foods and other products (Joshi and Pawar, 2015).

In addition, extracts from plants such as moringa, fennel, chamomile, rosemary and berries have been used as natural additives and preservatives in food to enhance organoleptic and nutritional qualities as well as extend the shelf life of products (Jayawardana et al., 2015; Caleja et al., 2016; Nieto et al., 2018; Lorenzo et al., 2018). There is also an on-going exploitation of plants as bio-pesticides for organic food production. For example, Raja and Masresha (2015) reported the effective application of plant compounds such as Azadirachtin obtained from the seed kernel of *Azadirachta indica* (Neem plant) and terpenoid esters found in dry flower buds of *Chrysanthimum* species in the control of pests.

In spite of the richness of plants in secondary metabolites and their diverse applications, some of them are considered toxic in humans. The level of toxicity displayed by each plant is dependent on the type of chemical compounds that constitute it (Nondo et al., 2015; Serrano, 2018). Moreover, the age, weight, health conditions of an exposed individual as well as part of the plant ingested, quantity of toxic compound(s) consumed and time interval between ingestion and manifestation of symptoms are factors that determine the degree of toxicity experienced (Serrano, 2018). The toxic effect of plants may vary from allergic reactions such as dermatitis (hives, itching, eczema, etc.), respiratory problems (cough, nasal congestion,

shortness of breath, etc.), gastrointestinal tract irritation (e.g. nausea, abdominal pain, vomiting, diarrhoea, etc.), to destruction of red blood cells, impairment of vital body organs (brain, kidney, heart, lungs and liver), and even death in severe cases (Nondo et al., 2015). Therefore, as safety precautions, it is crucial to determine the cytotoxicity of plants and plant-derived compounds on normal mammalian cells prior to their inclusion in any preservative formulations or products.

According to Liu et al. (2018) and Adefegha et al. (2018), plants like *Moringa oleifera* and *Xylopia aethiopica* are rich in secondary metabolites that are useful in preventing and treating different health conditions. They are also used to improve the nutritional and sensory qualities of food. In addition, previous chapters of this study (Chapter three and four) have demonstrated that the extracts of *M. oleifera* and *X. aethiopica* have antioxidant activities, anti-yeast properties against *Rhodotorula dairenensis, Wickerhamomyces anomalus, Zygosaccharomyces bailii* CY0757 and IGC4242, *Lodderomyces elongisporus* and *Cryptococcus laurentii* and growth stimulatory properties for *Yarrowia lipolytica*. This study was therefore carried out to evaluate the *in vitro* cytotoxicity of extracts from *M. oleifera* and *X. aethiopica*.

5.3. Methods

5.3.1. *In vitro* cytotoxicity of *M. oleifera* and *X. aethiopica* extracts

The extracts of *M. oleifera* and *X. aethiopica* were prepared from ground leaves and fruits respectively and as described in Chapter Three (section 3.3.1). The *in vitro* cytotoxicity of the plant extracts was evaluated using the Hoechst 33342 and propidium iodide (PI) dual staining procedure as described by Swanepoel et al. (2019). The methanol, acetone and water extracts of *M. oleifera* and *X. aethiopica* were reconstituted in dimethyl sulfoxide (DMSO) to give a final concentration of 100 mg/mL and stored at 4°C until required for further use.

5.3.1.1. Cell culture conditions

The African green monkey kidney cell line (Vero) was purchased from Cellonex, South Africa and maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) at 37°C in a humidified incubator with 5% CO_2 in 10 cm culture dishes.

5.3.1.2. Cell treatment procedure

The assay was carried out in quadruplicate as a single experiment in a 96-well micro-titre plate. A multi-channel pipette was used to distribute 100 µL of cells into column 1 to 8 at a density of 4000 cells per well. The plates were incubated at 37°C, 5% CO_2, and 100% relative humidity for 24 hours in order to allow for cell attachment before the addition of plant extracts. After incubation, 100 µL aliquots of plant extracts diluted in culture medium to give 100, 200 and 400 µg/mL concentrations were used to treat cells and then incubated for a further 48 hours. Wells in column 7 contained untreated cells which served as negative controls, Melphalan (40 µM), a standard drug, was used as positive control in column 8 while column 9 consisted of only medium which served as the blank.

Treatment medium was aspirated from all wells, replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL in phosphate-buffered saline) and incubated for 10 minutes at room temperature. Cells were then stained with propidium iodide (PI) at 100 µg/mL in order to enumerate the proportion of non-viable cells within the population. Cells were viewed immediately after the addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices) with a 10x Plan Fluor objective and DAPI and Texas Red filter cubes. Nine image sites were acquired per well which is representative of roughly 75% of the surface area of the well. Viable cells were stained with only the Hoechst 33342 while non-viable cells retained both Hoechst 33342 and PI dye. As a general guideline, plant extracts were considered potentially toxic if the number of viable cells in the presence of the extract is half or less than half of the untreated control population.

5.3.1.3. Data quantification and analysis

Quantification of viable and non-viable cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices). The obtained images were analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Data analysis and processing was carried out on Microsoft Excel spread sheet.

5.4. Results and discussion

5.4.1. *In vitro* cytotoxicity of *M. oleifera* and *X. aethiopica* extracts

Figure 5.1 depicts the cytotoxicity of *M. oleifera* and *X. aethiopica* extracts which were tested on the African green monkey kidney cell lines (Vero) using the Hoechst 33342 and propidium iodide (PI) dual staining method. The result indicated that the number of cells that remained viable after treating with *M. oleifera* extracts was more than half the number of viable cells in the untreated control. In other words, the number of viable cells was higher than the number of non-viable cells for all the extracts of *M. oleifera* at 100, 200 and 400 µg/mL concentrations.

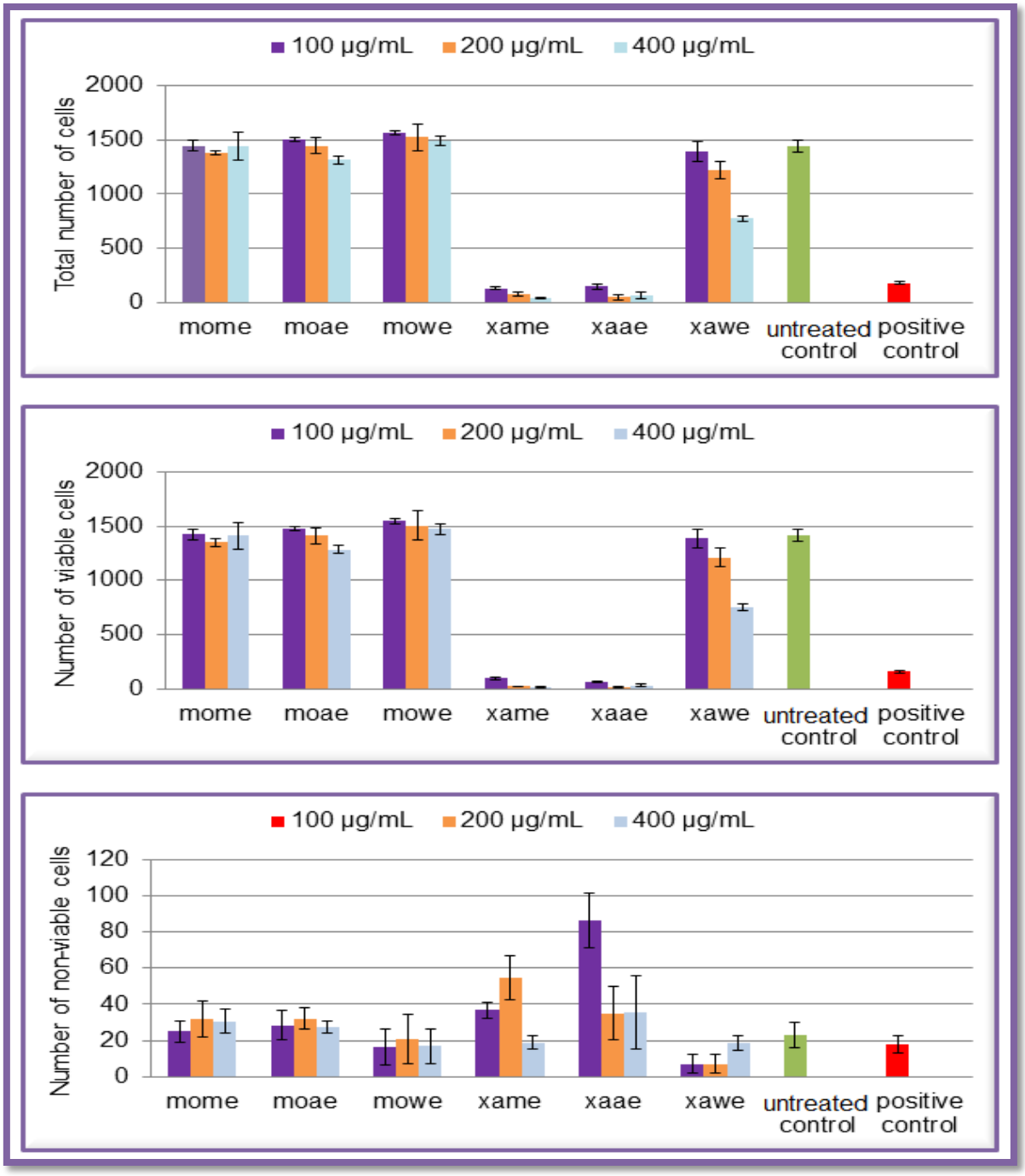

Figure 5.1. Total number of cells (top), number of viable cells (middle), number of non-viable cells (bottom). Error bars indicate SD of quadruplicate values performed as a single experiment. **Mome:** *M. oleifera* methanol extract, **Moae:** *M. oleifera* acetone extract, **Mowe:** *M. oleifera* water extract, **Xame:** *X. aethiopica* methanol extract, **Xaae:** *X. aethiopica* acetone extract, **Xawe:** *X. aethiopica* water extract

This implies that the leaf extracts of *M. oleifera* had no toxic effect on the cells even at the highest concentration (400 µg/mL). The non-cytotoxicity of *M. oleifera* leaf extracts on normal mammalian cells has already been documented (Charoensin, 2014; Abass et al., 2015; Rahaman et al., 2017).

Although some studies found low toxicity for *M. oleifera* leaf extracts, its safety on humans remains uncontested. For example, Jung (2014) reported that the cold water extract of *M. oleifera* leaves at 200 µg/mL displayed low toxicity on normal COS-7 cells derived from monkey kidney tissue. He further states that *M. oleifera* is not harmful on humans since more than 50% cell viability was observed for cells exposed to higher concentrations of the extract (600 µg/mL). Mansour et al. (2019) investigated the cytotoxicity of two Moringa species (*M. peregrina* and *M. oleifera*) on HFB4, a normal melanocytes cell line, using the Sulforhodamine B (SRB) assay. They discovered that the extracts of Moringa leaves exhibited low toxicity with cell viability ranging from 75 - 87% for *M. peregrina* and 80 - 87% for *M. oleifera.* The authors concluded that the decrease in cell survival after treatment with *M. oleifera* leaf extracts was insignificant. This indicates that the extracts from *M. oleifera* leaves are safe for human consumption and may be used as natural preservatives in fruit juice.

According to Figure 5.1, the methanol and acetone extracts of *X. aethiopica* at all concentrations (100, 200 and 400 µg/mL) were toxic with cell viability decreasing to less than half of the untreated control. It was also noted that the number of non-viable cells exceeded the number of viable cells. This supports Choumessi et al. (2012) who reported that at 25 µg/mL the ethanol extract of *X. aethiopica* was toxic on normal fibroblast cells. This finding was, however, different to the water extract which did not show any cytotoxicity to the cells at 400 µg/mL, indicating that only organic solvents can extract cytotoxic compound(s) from *X. aethiopica.* It is known that water is used for the preparation of food and extraction of bioactive compounds from plants in traditional medicines (Komape et al., 2017). Therefore, the non-cytotoxic effect of *X. aethiopica* water extract is crucial, bearing in mind that *X.*

aethiopica fruits are used to add flavour to different local dishes (Adefegha et al., 2018). This implies that only the water extract of *X. aethiopica* may be incorporated into fruit juices to extend their shelf life.

The cytotoxicity exhibited by the methanol and acetone extracts of *X. aethiopica* (Figure 5.1) may be attributed to the phytochemicals present in the extracts (Chapter three) or other compounds that were not screened for. In order to be sure of the main cause of toxicity shown by the extracts, isolation and identification of cytotoxic compound(s) are necessary. Also, further analysis needs to be undertaken to determine if the cytotoxic compound(s) are responsible for the antioxidant and anti-yeast activity observed in previous chapters (Chapter three and four). It is possible that the compound(s) showing cytotoxicity are not the same as the ones with the antioxidant and anti-yeast activity. In that case, the methanol and acetone extracts of *X. aethiopica* may still be used to control the growth of *R. dairenensis, Z. bailii* CY0757 and IGC4242, *W. anomalus, L. elongisporus* and *C. laurentii* in fruit juice (Chapter four), provided that the cytotoxic compound(s) are removed from the extracts.

5.5. Conclusion

This study has demonstrated that the water extract of *X. aethiopica* fruits as well as the methanol, acetone and water extract of *M. oleifera* leaves are not toxic on normal (Vero) cells *in vitro*. Thus, these extracts may be considered safe for human consumption. There are concerns regarding the safety of the methanol and acetone extracts of *X. aethiopica* on humans considering the massive decrease in cell viability when compared to the untreated control after a 48-hour period. However, these extracts may be potentially harmless and useful if, in the absence of the cytotoxic compounds, they still exhibit antioxidant and anti-yeast activity. Therefore, further investigations on the methanol and acetone extract of *X. aethiopica* are required.

5.6. References

Abass, M.A., Kabbashi, A.S. and Garbi, M.I. 2015. *In vitro* antioxidant activity, phytochemical analysis and cytotoxicity of ethanolic leaves extract of *Moringa oleifera* Lam. *International Journal of Multidisciplinary Research and Development,* 2(11): 73-77.

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Caleja, C., Barros, L., Antonio, A.L., Carocho, M., Oliveira, M.B.P.P. and Ferreira, I.C.F.R. 2016. Fortification of yogurts with different antioxidant preservatives: A comparative study between natural and synthetic additives. *Food Chemistry,* 210: 262-268.

Charoensin, S. 2014. Antioxidant and anticancer activities of *Moringa oleifera* leaves. *Journal of Medicinal Plant Research,* 8(7): 318-325.

Choumessi, A.T., Danel, M., Chassaing, S., Truchet, I., Penlap, V.B., Pieme, A.C., Asonganyi, T., Ducommun, B. and Valette, A. 2012. Characterization of the antiproliferative activity of *Xylopia aethiopica. Cell Division*, 7(1): 8.

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S. and Weththasinghe, P. 2015. Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. *LWT - Food Science and Technology*, 64: 1204-1208.

Joshi, L.S. and Pawar, H.A. 2015. Herbal cosmetics and cosmeceuticals: An overview. *Natural Products Chemistry and Research,* 3(2): 170.

Jung, I.L. 2014. Soluble extract from *Moringa oleifera* leaves with a new anticancer activity. *PLoS ONE*, 9(4): e95492.

Komape, N.P.M., Bagla, V.P., Kabongo-Kayoka, P. and Masoko, P. 2017. Antimycobacteria potential and synergistic effects of combined crude extracts of selected medicinal plants used by Bapedi traditional healers to treat

tuberculosis related symptoms in Limpopo Province, South Africa. *BMC Complementary and Alternative Medicine,* 17: 128.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

Lorenzo, J.M., Pateiro, M., Domínguez, R., Barba, F.J., Putnik, P., Kovačević, D.B., Shpigelman, A., Granato, D. and Franco, D. 2018. Berries extracts as natural antioxidants in meat products: A review. *Food Research International,* 106: 1095-1104.

Mansour, M., Mohamed, M.F., Elhalwagi, A., El-Itriby, H.A., Shawki, H.H. and Abdelhamid, I.A. 2019. *Moringa peregrina* leaves extract induce apoptosis and cell cycle arrest of hepatocellular carcinoma. *BioMed Research International,* 2019: 1-13.

Moosavi, M.A., Haghi, A., Rahmati, M., Taniguchi, H., Mocan, A., Echeverría, J., Gupta, V.K., Tzvetkov, N.T. and Atanasov, A.G. 2018. Phytochemicals as potent modulators of autophagy for cancer therapy. *Cancer Letters*, 424: 46-69.

Nieto, G., Ros, G. and Castillo, J. 2018. Antioxidant and antimicrobial properties of rosemary (*Rosmarinus officinalis*, L.): A review. *Medicines*, 5: 98.

Nondo, R.S.O., Moshi, M.J., Erasto, P., Zofou, D., Njouendou, A.J., Wanji, S., Ngemenya, M.N., Kidukuli, A.W., Masimba, P.J. and Titanji, V.P.K. 2015. Evaluation of the cytotoxic activity of extracts from medicinal plants used for the treatment of malaria in Kagera and Lindi regions, Tanzania. *Journal of Applied Pharmaceutical Science*, 5(4): 007-012.

Rahaman, M.H.A., Kadir, N.H.A., Amin, N.M. and Omar, W.B.W. 2017. Cytotoxicity effect of water extracts of *Moringa oleifera* leaves and seeds against MCF-7 cells. *Acta Horticulturae*, 1158: 279-286.

Raja, N. and Masresha, G. 2015. Plant based biopesticides: safer alternative for organic food production. *Journal of Fertilizers and Pesticides*, 6(2): e128.

Serrano, R. 2018. Toxic Plants: Knowledge, medicinal uses and potential human health risks. *Environment and Ecology Research,* 6(5): 487-492.

Soliman, S.S.M., Semreen, M.H., El-Keblawy, A.A., Abdullah, A., Uppuluri, P. and Ibrahim, A.S. 2017. Assessment of herbal drugs for promising anticandida activity. *BMC Complementary and Alternative Medicine,* 17: 257.

Swanepoel, B., Venables, L., Olaru, O.T., Nitulescu, G.M. and Venter, M.V. 2019. *In vitro* antiproliferative activity and mechanism of action of *Anemone nemorosa. International Journal of Molecular Sciences,* 20(5): 1217.

Chapter Six

General discussion, conclusions and recommendations

6.1. Discussion

Preservation which entails maintaining quality and extending shelf life of products by inhibiting microbial and enzymatic activity is a crucial part of quality assurance in the fruit juice industry (Karim et al., 2018). With the recent upsurge in interest for natural foods, plant-based compounds have become the focus of exploration as bio-preservatives in food. This research was carried out to investigate the potential of *Moringa oleifera* and *Xylopia aethiopica* extracts for use as preservatives in grape and orange juice. In order to achieve this aim, the following tests were carried out on the plant extracts and results were reported accordingly;

1. phytochemical screening (Chapter three)
2. antioxidant activity (Chapter three)
3. total phenolic contents (Chapter three)
4. anti-yeast activity (Chapter four)
5. microbiological analysis of treated and untreated fruit juices (Chapter four)
6. cytotoxicity (Chapter five)

A summary of the results is presented in Table 6.1. The phytochemicals found in the extracts from *M. oleifera* have been reported to have many biological effects like antiinflammatory, antidiabetic, antimicrobial, antioxidant and anticancer (Khan et al., 2018; Moosavi et al., 2018). This justifies the use of *M. oleifera* in the treatment of various diseases and in the fortification of food (Al_husnan and Alkahtani, 2016; Bolarinwa et al., 2019). All the extracts from *X. aethiopica* showed the presence of various phytochemicals (Table 6.1). The presence of these chemical compounds and their diverse concentrations in the extracts may explain the differences in the bioactivities observed in Chapter three and four.

Table 6.1. Summary of bioassay results for *M. oleifera* and *X. aethiopica* extracts

	M. oleifera			***X. aethiopica***		
	Methanol	**Acetone**	**Water**	**Methanol**	**Acetone**	**Water**
Phytochemical contents	Alkaloids, saponins, tannins, flavonoids, glycosides, terpenoids	Alkaloids, saponins, tannins, flavonoids, glycosides, terpenoids	Saponins, tannins, flavonoids, glycosides, terpenoids	Alkaloids, tannins,saponins, flavonoids, glycosides	Alkaloids, tannins, saponins, flavonoids, glycosides	Alkaloids, tannins, saponins, flavonoids, glycosides
Antioxidant activity (IC_{50} µg/mL)	48.09	86.54	124.5	31.02	19.10	40.31
Total phenolic content (mg GAE/g)	110.0	97.77	68.54	132.4	173.2	93.92
Anti-yeast activity	Fungicidal activity for *W. anomalus, Z. bailii* CY0757 and IGC4242 at 10, 2.5 and 5 mg/mL respectively. Fungistatic activity for *W. anomalus* at 5 mg/mL and *Z. bailii* IGC4242 at 2.5 mg/mL. Stimulatory activity for *Y. lipolytica.*	No fungicidal or fungistatic activity. Stimulatory activity for *Y. lipolytica.*	No fungicidal or fungistatic activity. Stimulatory activity for *Y. lipolytica.*	Fungicidal activity for *Z. bailii* IGC4242 at 2.5 mg/mL and *C. laurentii* at 5 mg/mL Fungistatic activity for *Z. bailii* IGC4242 at 1.25 mg/mL, *C. laurentii* at 1.25 mg/mL, *W. anomalus, Z. bailii* CY0757, *R. dairenensis* and *L. elongisporus* at 10 mg/mL.	No fungicidal activity. Fungistatic activity for *Z. bailii* IGC4242 at 1.25 mg/mL and *C. laurentii* at 5 mg/mL.	No fungicidal or fungistatic activity.
Microbiological analysis	Overgrowth	Not tested	Not tested	Overgrowth	Not tested	Not tested
Cytotoxicity	Not toxic at all concentrations	Not toxic at all concentrations	Not toxic at all concentrations	Toxic at all concentrations	Toxic at all concentrations	Not toxic at all concentrations

The plant extract with the best antioxidant activity also had the highest total phenolic content (Chapter three). This confirms that antioxidant activity of plant extracts are mainly attributed to phenolic compounds present (Yao et al., 2016; Guldiken et al., 2018). The highest antioxidant activity and total phenolic content for *M. oleifera* was exhibited by the methanol extract. This suggests that *M. oleifera* methanol extract has the potential of inhibiting oxidation in fruit juices and increasing the antioxidant capacity of fruit juices. This result supports Jayawardana et al. (2015) who demonstrated that *M*. *oleifera* leaves can inhibit lipid oxidation in chicken sausages. It also corroborates other studies that have reported that *M. oleifera* leaves is useful in the fortification of yoghurt, cookies and bread (Kuikman and O'Connor, 2015; Haneen, 2015; Bolarinwa et al., 2019).

Although the highest antioxidant activity and total phenolic content for *X. aethiopica* was seen in the acetone extract, the methanol and water extract also exhibited good antioxidant activity and total phenolic content (Chapter three). This indicates that the methanol and water extract of *X. aethiopica* may still function as natural antioxidants and inhibit oxidation in fruit juices even though the acetone extract showed the highest antioxidant activity and total phenolic content. These results support Bode and Dong (2015) who documented that herbs and spices have the potential of extending the shelf life of foods owing to their antioxidant and antimicrobial properties. Moreover, the results validate the value of spices such as *X. aethiopica* fruits for enhancing nutritional and sensory characteristics of food and for medicinal purposes (Ngwoke et al., 2015; Adefegha et al., 2018).

The plant extracts were tested against *Saccharomyces cerevisiae* ATCC26602 and IGC3507, *Zygosaccharomyces bailii* CY0757 and IGC4242, *Yarrowia lipolytica, Candida parapsilosis, Wickerhamomyces anomalus, Lodderomyces elongisporus, Rhodotorula dairenensis* and *Cryptococcus laurentii* in order to evaluate their anti-yeast properties (Chapter four). These yeasts were selected based on their involvement in fruit juice spoilage. The methanol extract from *M. oleifera* performed better than the acetone and water extract in terms of its fungicidal and fungistatic

activity against certain spoilage yeasts (Table 6.1). The acetone and water extract did not reveal much bioactivity against the yeasts; thus they were considered as weak growth inhibitors. This was expected for the water extract since water is not very efficient in extracting bioactive molecules from plants (Dzoyem et al., 2016). However, the acetone extract showing little to no anti-yeast activity was unforeseen because acetone has been reported as the best solvent for extracting antimicrobial compounds from plants (Dzoyem et al., 2016). As previously mentioned in Chapter four, polarity of solvent is a major contributor to the amount of bioactive compounds derived from plants (Wanyo et al., 2016). Therefore, the poor performance of acetone extract may be attributed to the extracted compounds with anti-yeast activities that were not soluble in 50 % acetone used for extraction. These findings indicate that only the methanol extract from *M. oleifera* may be used to inhibit the growth of some yeasts associated with fruit juice spoilage (Table 6.1).

An interesting observation that was far from the much anticipated anti-yeast activity was made for *M. oleifera*. All its extracts seemed to stimulate the growth of *Y. lipolytica* with increasing concentration and time, implying that the yeast utilises *M. oleifera* as substrate for growth. It is important to note that *Y. lipolytica* thrives in hydrophobic environments by making use of *n*-alkanes, fats, oils and fatty acids as carbon sources (Gonçalves et al., 2014) and that *M. oleifera* leaves have abundant unsaturated fatty acids (Liu et al., 2018). This may explain the stimulatory activity of *M. oleifera* leaf extracts on *Y. lipolytica* (Chapter four). This indeed is a remarkable observation as *Y. lipolytica* is useful in the production of biofuel (Darvishi et al., 2017).

Again, only the methanol extract displayed fungicidal activity among the extracts of *X. aethiopica* (Table 6.1). The methanol extract also showed fungistatic activity and inhibition less than 50 % against some yeasts (Chapter four). Although no fungicidal effect was observed by the acetone extract of *X. aethiopica* it revealed fungistatic activity that was manifested as 100% inhibition after 72 hours against *Z. bailii* IGC4242 and *C. laurentii.* The acetone extract also showed inhibitory activity within

the ranges of 22.4 - 76.6% against *R. dairenensis, W. anomalus* and *Y. lipolytica* after 120 hours (Chapter four). The water extract was the least active of all the extracts. This indicates that the methanol extract of *X. aethiopica* can be used as both fungicidal and fungistatic agent for certain fruit juice spoilage yeasts while the acetone extract can only function as growth inhibitor for a specific period of time (Chapter four). It was presumed that extracts from *X. aethiopica* would show some inhibitory activity against the yeasts considering the reports on its medicinal values (Ngwoke et al., 2015; Adefegha et al., 2018). Also, Ogbonna et al. (2013) reported the antimicrobial activity of *X. aethiopica* against some food spoilage and pathogenic organisms.

The results for the anti-yeast activity test confirms that 50% acetone used for extracting the plant materials minimised the concentration of anti-yeast compounds that were extracted from both plant species. The level of antioxidant and anti-yeast activity observed in Chapter three and four respectively, may be attributed to the fact that the extracts were used in crude forms. Crude extract from plants usually contain a combination of different compounds including chlorophyll, carbohydrates, sugars, fats and oils (Raks et al., 2018). It is possible that the extracts from *M. oleifera* and *X. aethiopica* may exhibit higher antioxidant and anti-yeast activity if, the active compounds are isolated and purified.

Researchers have suggested that extracts and essential oils from plants may be good alternatives for maintaining quality and safety of foods because of their antioxidant and antimicrobial properties (Calo et al., 2015; Tzima et al., 2015). It has also been demonstrated that plants like *M. oleifera* and *X. aethiopica* can be used to prolong the shelf life of foods (Ogbonna et al., 2013; Jayawardana et al., 2015). In this study, *M. oleifera* and *X. aethiopica* extracts did not exert inhibitory effect on some of the fruit juice spoilage yeasts and this disqualifies their use as natural antimicrobials in fruit juice. However, this does not mean that the plant extracts cannot be used as natural antimicrobial preservatives in other foods because a variety of microorganisms such as bacteria, yeasts and moulds are responsible for

causing spoilage in different foods. In other words, *M. oleifera* and *X. aethiopica* extracts may not have strong anti-yeast effects on certain fruit juice spoilage yeasts but they may have strong antimicrobial effects on other microorganisms that cause spoilage in foods.

Microbiological analysis showed that fruit juices treated with the methanol extract from *M. oleifera* and *X. aethiopica* contained microbial overgrowth that could not be enumerated. Nonetheless, due to the presence of contaminants in untreated fruit juices used as controls, the experiment was reported inconclusive in Chapter four. As earlier mentioned, the fruit juices might have been cross-contaminated with the microflora of fresh fruits before inoculating with the spoilage yeasts (Oluwole et al., 2016). Therefore, concluding that the plant extracts did not inhibit yeast growth in the grape and orange juice would be incorrect.

The methanol, acetone and water extracts from *M. oleifera* were not cytotoxic to the Vero cells (Chapter five), indicating the safety of the plant on humans. The results in Chapter three and four revealed that the methanol extract of *M. oleifera* exhibited the highest total phenolic content, antioxidant, anti-yeast and stimulatory activity. This implies that only the methanol extract from *M. oleifera* has the potential to inhibit oxidation and the growth of some fruit juice spoilage yeasts even though all the extracts were not toxic on the Vero cells (Chapter five).

The water extract from *X. aethiopica* exhibited zero toxicity to Vero cells (Table 6.1), suggesting its safety on humans. The methanol and acetone extracts, on the other hand, drastically reduced the number of viable cells after treatment, indicating that they might not be safe for human health (Chapter five). This means that only the water extract of *X. aethiopica* may be used in the preservation of fruit juice. However, owing to the fact that the water extract showed good antioxidant activity and poor anti-yeast activity (Chapter three and four), it may only serve as inhibitor of oxidation in fruit juices. According to Table 6.1, the acetone extract exhibited the highest antioxidant activity and total phenolic content, the methanol extract showed

the best anti-yeast activity while all the extracts except the water extract displayed cytotoxicity on Vero cells. This suggests that the secondary metabolites responsible for antioxidant activity in *X. aethiopica* fruits may be different from the ones with anti-yeast activity. This highlights the need for isolation and purification of compounds with antioxidant and anti-yeast characteristics from the plant. It is also necessary to analyse the cytotoxicity of these compounds so as to know if they are responsible for the cytotoxicity of the methanol and acetone extract.

6.2. Conclusions

This research has demonstrated that the methanol extract of *M. oleifera* leaves has the potential to slow down oxidation in fruit juice due to its high antioxidant activity and total phenolic content. It has also been demonstrated that the methanol extract of *M. oleifera* can inhibit the growth of some fruit juice spoilage yeasts *in vitro*. Moreover, all the extracts from *M. oleifera* leaves are not toxic on normal Vero cells. However, *M. oleifera* extracts may not have the potential as natural antimicrobial preservatives for fruit juice because they did not inhibit some of the tested yeasts. *X. aethiopica* fruits contain phenolic compounds with good antioxidant activity that may be exploited as natural antioxidants in fruit juice. The methanol and acetone extract have inhibitory activity that can be used to control the growth of certain yeasts associated with fruit juice spoilage. Nevertheless, due to the insensitivity of some of the yeasts toward the methanol and acetone extract of *X. aethiopica* and their intense toxicity on normal Vero cells, it is concluded that these extracts cannot be used in the preservation of fruit juice.

6.3. Recommendations

It is recommended to wash thoroughly fresh fruits with clean sterile water and sanitise them before processing into other forms to avoid cross-contamination. It is also recommended to practice aseptic techniques throughout the handling processes. The growth stimulatory activity of *M. oleifera* extracts on *Yarrowia lipolytica* warrants further investigation as the yeast is useful in the production of biofuels. *M. oleifera* and *X. aethiopica* exhibited potential as natural antioxidants in

fruit juice. It is thus recommended to investigate oxidation of fruit juices in future experiments and the application of the plant extracts on other fruit juices like apple juice, as this may prevent colour changes. Finally, isolation and identification of compound(s) responsible for the various activities observed in the extracts of *M. oleifera* and *X. aethiopica* is recommended. This is to verify that the extracts would exhibit better antioxidant, anti-yeast or stimulatory activity after purification and confirm if the methanol and acetone extract from *X. aethiopica* would show toxicity in their purified state.

6.4. References

Adefegha, S.A., Oboh, G., Olasehinde, T.A. and Boligon**,** A.A. 2018. Dietary supplementation with Ethiopian pepper (*Xylopia aethiopica*) modulates angiotensin-I converting enzyme activity, antioxidant status and extenuates hypercholesterolemia in high cholesterol fed Wistar rats. *PharmaNutrition,* 6: 9-16.

Al_husnan, L.A. and Alkahtani, M.D.F. 2016. Impact of moringa aqueous extract on pathogenic bacteria and fungi *in vitro*. *Annals of Agricultural Science,* 61: 247-250.

Bode, A.M. and Dong, Z. 2015. Toxic phytochemicals and their potential risks for human cancer. *Cancer Prevention Research,* 8(1): 1-8.

Bolarinwa, I.F., Aruna, T.E. and Raji, A.O. 2019. Nutritive value and acceptability of bread fortified with moringa seed powder. *Journal of the Saudi Society of Agricultural Sciences*, 18: 195-200.

Calo, J.R., Crandall, P.G., O'Bryan, C.A. and Ricke**,** S.C. 2015. Essential oils as antimicrobials in food systems: A review. *Food Control,* 54: 111-119.

Darvishi, F., Fathi, Z., Ariana, M. and Moradi, H. 2017. *Yarrowia lipolytica* as a workhorse for biofuel production. *Biochemical Engineering Journal,* 127: 87-96.

Dzoyem, J.P., Aro, A.O., McGaw, L.J. and Eloff, J.N. 2016. Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal

plants used in southern Africa to treat tuberculosis and respiratory ailments. *South African Journal of Botany,* 102: 70-74.

Gonçalves, F.A.G., Colen, G. and Takahashi, J.A. 2014. *Yarrowia lipolytica* and its multiple applications in the biotechnological industry. *The Scientific World Journal*, 2014: 1-14.

Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F.D., Yalcinkaya, I.E. and Capanoglu, E. 2018. Phytochemicals of herbs and spices: health versus toxicological effects. *Food and Chemical Toxicology,* 119: 37-49.

Haneen, H.S.M. 2015. Effect of dried *Moringa oleifera* leaves on the nutritional and organoleptic characteristics of cookies. *Alexandria Science Exchange Journal,* 36(4): 297-305.

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S. and Weththasinghe, P. 2015. Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. *LWT - Food Science and Technology*, 64: 1204-1208.

Karim, N.U., Siddiq, U.S.A.A., Razak, M.R.M., Zainol, M.K.M., and Abdullah, M.I. 2018. Effects of moringa leaves (*Moringa oleifera*) extraction on quality changes and melanosis of giant freshwater prawn (*Macrobrachium rosenbergii*) during chilled storage. *Italian Journal of Food Safety,* 7(3): 6846.

Khan, H., Amin, S. and Patel, S. 2018. Targeting BDNF modulation by plant glycosides as a novel therapeutic strategy in the treatment of depression. *Life Sciences*, 196: 18-27.

Kuikman, M. and O'Connor, C.P. 2015. Sensory evaluation of moringa-probiotic yogurt containing banana, sweet potato or avocado. *Journal of Food Research*, 4: 165-171.

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T. and Han, J.P. 2018. Values, properties and utility of different parts of *Moringa oleifera*: An overview. *Chinese Herbal Medicines,* 10: 371-378.

Moosavi, M.A., Haghi, A., Rahmati, M., Taniguchi, H., Mocan, A., Echeverría, J., Gupta, V.K., Tzvetkov, N.T. and Atanasov, A.G. 2018. Phytochemicals as

potent modulators of autophagy for cancer therapy. *Cancer Letters*, 424: 46-69.

Ngwoke, K.G., Ikeanyi, A.U., Eze, P.M., Ezemokwe, I.C., Abba, C.C. and Ugwu, M.C. 2015. Phytochemical and antioxidant properties of extracts of *Xylopia aethiopica* fruits. *Chemical Science Review and Letters*, 4(13): 267-270.

Ogbonna C.N., Nozaki, K. and Yajima, H. 2013. Antimicrobial activity of *Xylopia aethiopica*, *Aframomum melegueta* and *Piper guineense* ethanolic extracts and the potential of using *Xylopia aethiopica* to preserve fresh orange juice. *African Journal of Biotechnology,* 12(16): 1993-1998.

Oluwole, O.A., David, O.M., Falegan, C.R., Awojuyigbe, B. and Olajide, O.M. 2016. Microbiological and physicochemical properties of commercial seal tampered refrigerated fruit juices. *International Journal of Biological Research,* 4(1): 21-24.

Raks, V., Al-Suod, H. and Buszewski, B. 2018. Isolation, separation and preconcentration of biologically active compounds from plant matrices by extraction techniques. *Chromatographia*, 81: 189-202.

Tzima., K., Makris, D., Nikiforidis, C.V. and Mourtzinos I. 2015. Potential use of rosemary, propolis and thyme as natural food preservatives. *Journal of Nutrition and Health,* 1(1): 6.

Wanyo, P., Kaewseejan, N., Meeso, N. and Siriamornpun, S. 2016. Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by-products. *Applied Biological Chemistry,* 59(3): 373-384.

Yao, X.H., Zhang, Z.B., Song, P., Hao, J.Y., Zhang, D.Y. and Zhang, Y.F. 2016. Different harvest seasons modify bioactive compounds and antioxidant activities of *Pyrola incarnata. Industrial Crops and Products,* 94: 405-412.

Appendix

Research article

Baah, G.O., Manduna, I.T., Malebo, N.J. and De Smidt, O. (in press). Is *Moringa oleifera* a potential natural preservative for fruit juice? *Acta Horticulturae*.

Is *Moringa oleifera* a potential natural preservative for fruit juice?

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Abstract

Most fruit juice preservatives are chemically synthesised, and knowledge of the health risks associated with their consumption has resulted in the search for safer alternatives, such as secondary plant metabolites. This study evaluated the phytochemical and anti-yeast properties of acetone, methanol and water extracts from *Moringa oleifera* in order to explore their potential for use as preservatives in fruit juice. Qualitative phytochemical analysis indicated the presence of alkaloids, flavonoids, tannins, terpenoids, glycosides and saponins in all extracts except the water extract which lacked alkaloids. The extracts were tested against ten yeast isolates involved in fruit juice spoilage using the agar dilution assay. All plant extracts inhibited *Rhodotorula dairenensis* and stimulated the growth of *Yarrowia lipolytica* at 0.625 mg/mL. The methanol extract was fungicidal against *Wickerhamomyces anomalus* at 10 mg/mL, the CY0757 strain of *Zygosaccharomyces bailii* at 2.5 mg/mL and the IGC4242 strain at 5 mg/mL. Growth of *Saccharomyces cerevisiae* (ATCC26602 and IGC3507), *Lodderomyces elongisporus, Cryptococcus laurentii* and *Candida parapsilosis* was not affected by the extracts. The acetone and water extracts exhibited the least anti-yeast activity. While *M. oleifera* extracts contain various secondary metabolites with anti-yeast properties, this study has demonstrated their limited potential as natural preservatives in fruit juice. However, the growth stimulatory activity of the extracts on *Yarrowia lipolytica* may be useful in the production of biofuels and warrants further investigation.

Keywords: Beverage, spoilage, anti-yeast activity, secondary plant metabolites, preservation.

INTRODUCTION

Nutritious and ready to serve beverages such as fruit juices have increasingly become a household drink in many parts of the world due to their richness in sugars, vitamins, antioxidants and other bioactive compounds with health promoting properties. Nevertheless, their abounding nutrients and high sugar contents favour microbial growth, thus making them highly susceptible to spoilage (Cardador and Gallego, 2015; Soto et al., 2019). Although a variety of microorganisms (acid tolerant bacteria, yeasts and moulds) can cause spoilage in fruit juices, yeasts are the primary contaminants of fresh and concentrated fruit juices, mainly because many of them can grow anaerobically and in high acidic environments (Bukvicki et al., 2014).

Pasteurisation and chemical preservatives have been conventionally used to maintain the quality and safety of fruit juices. Even so, their applications are met with different drawbacks. For example, changes in sensory qualities (aroma, flavour and colour) and loss of nutritional properties (vitamins, carotenoids, polyphenols, etc.) may occur in fruit juices that have been subjected to heat treatment. Likewise, studies have reported that constant exposure to the chemical compounds e.g. sulphur dioxide, benzoic and sorbic acids used in the preservation of

fruit juices may produce allergic, neurotoxic, carcinogenic and other adverse effects on human health (Guedes et al., 2016; Ng et al., 2019). Consequently, various approaches such as ultrasound, pulsed electric field, UV-C light, and the application of natural compounds from plant sources are continuously being researched as alternatives to ensure the safety and quality of fruit juices (Almeida et al., 2019).

Moringa oleifera Lam. (Moringaceae) is a multipurpose plant that contains a variety of phytochemicals that are useful in water purification, medicine, cosmetics, as biopesticides, etc. (Djande et al., 2018). The potential use of *M. oleifera* as a natural preservative as well as its use in the enhancement of sensory and nutritional qualities of food has also been reported (Haneen, 2015; Jayawardana et al., 2015; Gull et al., 2016; Bolarinwa et al., 2019). This study was conducted to evaluate the phytochemical and anti-yeast properties of *M. oleifera* extracts in order to explore their potential for use as preservatives in fruit juice.

MATERIALS AND METHODS

Preparation of extracts

M. oleifera leaf powder was purchased from Limpopo, South Africa. Fifty grams of dry powder was separately macerated in 250 mL of 50 % acetone, 50 % methanol and distilled water and kept at room temperature for three days with continuous shaking. The mixture was filtered and re-extracted every 12 hours. The extracts were concentrated by removing the solvents using a Genevac Rocket evaporator system while the water extract was lyophilised. The plant extracts were weighed and stored in the refrigerator at 4˚C until further analysis. The percentage extract yields were calculated using the following formula:

$$\text{Extract yields (\%)} = \frac{\text{mass of extract obtained (g)}}{\text{mass of plant sample used (g)}} \text{x } 100$$

Phytochemical screening

Standard qualitative methods were used to screen for the presence of tannins, flavonoids, terpenoids, alkaloids, saponins and glycosides in the extracts (Al Ghasham et al., 2017).

In vitro anti-yeast activity of *M. oleifera* extracts

1. Yeast and culture conditions

Ten different yeasts involved in fruit juice spoilage were used in this study. *Lodderomyces elongisporus*, *Rhodotorula dairenensis*, *Wickerhamomyces anomalus*, *Yarrowia lipolytica*, *Candida parapsilosis* and *Cryptococcus laurentii* were obtained from the Centre for Applied Food Sustainability and Biotechnology (CAFSaB) Central University of Technology, Free State culture collection. These specimens were isolated from the production environment and spoiled product of a fruit juice production facility (Corbett and de Smidt, 2019). *Saccharomyces cerevisiae* (ATCC26602 and IGC3507) and *Zygosaccharomyces bailii* (CY0757 and IGC4242) were sourced from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free State. The yeast cultures were maintained at -20˚C in Yeast extract Peptone Dextrose (YPD) broth containing 15 % glycerol.

2. Agar dilution assay

The anti-yeast activity of *M. oleifera* extracts was determined using the agar dilution method according to López-Carballo et al. (2012). The plant extracts were dissolved in 30 mL of

sterile distilled water, 1 % acetone and 1 % methanol to make an initial concentration of 20, 10, 5, 2.5 and 1.25 mg/mL. Subsequently, 30 mL of warm molten Potato Dextrose Agar (PDA) was added to the extracts and gently swirled. The mixture with a final concentration of 10, 5, 2.5, 1.25 and 0.625 mg/mL was poured into petri dishes and allowed to solidify. A set of controls consisting of a blank (only PDA) and PDA mixed with 1 % acetone and 1 % methanol were prepared in order to ensure the non-inhibitory effect of the solvents at 1 % against the yeasts. All experiments were carried out aseptically and each test was performed in triplicate.

Yeast cultures were revived by sub-culturing on PDA plates and incubating at 28˚C for 48 hours. After incubation, well isolated colonies were transferred onto fresh PDA plates and incubated for a further 48 hours. Thereafter, cells from the 48 hour culture were added directly into 5 mL of sterile saline solution, standardized to 0.5 McFarland's turbidity with a spectrophotometer and inoculated into 20 mL of broth containing glucose and peptone. The inoculated broth was then loaded onto a MAST **Uri®***Dot* multipoint inoculator and cells were immediately distributed to the PDA plates at various points. The plates were incubated at 28˚C and yeast growth was recorded after 48, 72 and 120 hours. Diameter of yeast growth was measured with a Central 6420 Vernier Calliper and expressed as percentage growth inhibition using the formula:

$$\% \text{ inhibition} = \frac{\text{growth of yeast in control} - \text{growth of yeast in extract}}{\text{growth of yeast in control}} \text{x } 100$$

Statistical analysis

Data for anti-yeast activity was subjected to analysis of variance (ANOVA) using SAS software, where differences between treatment means were ranked using the Least Significance Difference test (LSD 0.05).

RESULTS AND DISCUSSION

Percentage yields of *M. oleifera* extracts

The methanol and water extracts of *M. oleifera* yielded a brownish powder while the acetone extract yielded a greenish, jelly-like mass. The acetone extract had the highest yield of 67.94 %, followed by methanol (59.92 %) and water extract (9.65g). The type and polarity of solvents may have contributed to the variation in colour and masses. Acetone being the most non-polar solvent used probably allowed the extraction of other non-polar compounds like chlorophyll, fats and oils thus affecting the colour and increasing the yield. Conversely, water having the highest polarity index may have extracted only polar compounds thereby resulting in low yield (Ngo et al., 2017).

Qualitative phytochemical content of *M. oleifera* extracts

The phytochemical constituents of *M. oleifera* leaf extracts are presented in Table 1 which also shows screening results from other studies. The contents of the water and methanol extracts of *M. oleifera* leaves were consistent with the findings of Vinoth et al. (2012) and Shanmugavel et al. (2018). However, the number and type of phytochemical groups present differed from those in other studies (Table 1.) These differences may be resultant from the aqueous-organic solvent mixtures (1:1) that were used for extraction. In addition, environmental factors from the source of plant material, such as the climatic conditions, soil type, sun exposure and geographical locations contribute to the kind and quantities of secondary metabolites present in plants (Kumar et al., 2017).

Table 1. Phytochemical contents of *M. oleifera* leaf extracts

	F	G	T	A	S	T
Methanol	+	+	+	+	+	+
Shanmugavel et al. (2018)	+	+	+	+	+	+
Maqsood et al. (2017)	+	-	-	+	+	+
Acetone	+	+	+	+	+	+
Padmalochana (2018)	+	+	+	-	-	+
Water	+	+	+	-	+	+
Vinoth et al. (2012)	+	+	+	-	+	+
Maqsood et al. (2017)	-	-	-	+	-	+
Patel et al. (2014)	+	-	-	+	+	-

F: Flavonoids, **G:** Glycosides, **T:** Terpenoids, **A:** Alkaloids, **S:** Saponins, **T:** Tannins, **+:** Positive test, **-:** Negative test.

Anti-yeast activity of *M. oleifera* extracts

Anti-yeast activity of *M. oleifera* as percentage growth inhibition of *W. anomalus, Z. bailii* CY0757 and IGC4242, *R. dairenensis* and *Y. lipolytica* are shown in Table 2. *L. elongisporus, C. laurentii, C. parapsilosis, S. cerevisiae* ATCC26602 and IGC3507 were least affected by the extracts over 120 hours with inhibition ranging from 0-38.3% and therefore not presented in Table 2. All the yeasts used were not susceptible to acetone and methanol at 1 % which were not significantly different from the control. Minimum inhibitory concentrations (MIC) were documented at 48hrs of treatment.

Table 2. Growth inhibition of yeast (%) after treating with plant extracts at different time intervals

Extract	Time (hours)	Concentration (mg/mL)	WA	ZB1	ZB2	RD	YL
Methanol	48	10	100.0^{p}	100.0^{n}	100.0^{m}	56.9^{q}	-75.5^{on}
		5	**100.0^{p}**	100.0^{n}	100.0^{m}	50.0^{p}	-55.1^{qr}
		2.5	0.0^{n}	100.0^{n}	100.0^{m}	37.9^{on}	-51.0^{qrs}
		1.25	0.0^{n}	42.5^{m}	20.6^{kj}	37.9^{on}	-42.9^{tus}
		0.625	0.0^{n}	**32.5^{kl}**	**14.7^{ij}**	**32.8^{mln}**	-36.7^{u}
		1 % Methanol	0.0^{n}	5.0^{gf}	0.0^{gf}	1.7^{ij}	2.0^{y}
		Control	0.0^{n}	0.0^{gf}	0.0^{gf}	0.0^{ij}	0.0^{y}
	72	10	100.0^{p}	100.0^{n}	100.0^{m}	68.2^{qp}	-106.8^{cd}
		5	47.1^{o}	100.0^{n}	100.0^{m}	65.9^{p}	-78.0^{hg}
		2.5	25.0^{ml}	100.0^{n}	100.0^{m}	54.1^{mln}	-78.0^{hg}
		1.25	25.0^{ml}	13.0^{gf}	31.0^{ij}	54.1^{mln}	-67.8^{ji}
		0.625	19.1^{kj}	13.0^{gf}	19.0^{gf}	50.6^{l}	-39.0^{op}
		1 % Methanol	1.5^{de}	0.0^{d}	0.0^{ed}	1.2^{b}	1.7^{wv}
		Control	0.0^{de}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}
	120	10	100.0^{p}	100.0^{n}	100.0^{m}	57.9^{gh}	-120.3^{a}
		5	32.9^{ml}	100.0^{n}	100.0^{m}	53.8^{fe}	-73.9^{d}
		2.5	19.7^{gh}	100.0^{n}	73.6^{l}	53.8^{fe}	-73.9^{d}
		1.25	11.8^{de}	16.7^{d}	15.1^{cb}	50.3^{c}	-65.2^{e}
		0.625	9.2^{dc}	14.8^{d}	13.2^{b}	50.3^{c}	-29.0^{mn}
		1 % Methanol	1.3^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}
		Control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}
Acetone	48	10	0.0^{n}	37.5^{ml}	26.5^{k}	50.0^{p}	22.4^{z}
		5	0.0^{n}	27.5^{kj}	20.6^{kj}	41.4^{o}	14.3^{z}

Extract	Time (hours)	Concentration (mg/mL)	WA	ZB1	ZB2	RD	YL
		2.5	0.0^{n}	27.5^{kj}	14.7^{ij}	37.9^{on}	2.0^{y}
		1.25	0.0^{n}	27.5^{kj}	**14.7^{ij}**	34.5^{mn}	-20.4^{wv}
		0.625	0.0^{n}	**25.0^{ij}**	5.9^{gh}	**34.5^{mn}**	-36.7^{u}
		1 % acetone	0.0^{n}	5.0^{gf}	5.9^{gh}	1.7^{ij}	2.0^{y}
		Control	0.0^{n}	0.0^{gf}	0.0^{gh}	0.0^{ij}	0.0^{y}
	72	10	19.1^{kj}	41.3^{kl}	28.6^{ih}	60.0^{o}	13.6^{yx}
		5	16.2^{ij}	34.8^{ij}	19.0^{gf}	57.6^{on}	6.8^{wx}
		2.5	16.2^{ij}	13.0^{gf}	19.0^{gf}	55.3^{mn}	-22.0^{trs}
		1.25	7.4^{gf}	13.0^{gf}	14.3^{f}	50.6^{l}	-42.4^{o}
		0.625	2.9^{de}	8.7^{ef}	14.3^{f}	50.6^{l}	-45.8^{on}
		1 % acetone	1.5^{de}	0.0^{d}	0.0^{ed}	1.2^{b}	1.7^{wv}
		Control	0.0^{de}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}
	120	10	14.5^{fe}	46.3^{kj}	22.6^{ed}	64.8^{k}	11.6^{v}
		5	9.2^{dc}	37.0^{h}	18.9^{cd}	60.7^{ij}	0.0^{tu}
		2.5	6.6^{bc}	14.8^{d}	18.9^{cd}	56.6^{g}	-42.0^{ji}
		1.25	2.6^{ba}	14.8^{d}	13.2^{b}	52.4^{dce}	-44.9^{i}
		0.625	1.3^{a}	9.3^{c}	11.3^{b}	51.0^{dc}	-46.4^{hi}
		1 % acetone	2.6^{a}	1.9^{a}	0.0^{a}	0.0^{a}	0.0^{tu}
		Control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}
Water	48	10	0.0^{n}	27.5^{kj}	14.7^{ij}	41.4^{o}	-59.2^{qp}
		5	0.0^{n}	20.0^{ih}	14.7^{ij}	37.9^{on}	-55.1^{qr}
		2.5	0.0^{n}	15.0^{h}	14.7^{ij}	34.5^{mn}	-46.9^{trs}
		1.25	0.0^{n}	15.0^{h}	11.8^{ih}	34.5^{mn}	-46.9^{trs}
		0.625	0.0^{n}	**15.0^{h}**	**11.8^{ih}**	**34.5^{mn}**	-42.9^{tus}
		Control	0.0^{n}	0.0^{gf}	0.0^{gf}	0.0^{ih}	0.0^{y}
		10	22.1^{kl}	17.4^{g}	14.3^{f}	52.9^{ml}	-79.7^{g}
	72	5	16.2^{ij}	13.0^{gf}	14.3^{f}	50.6^{l}	-64.4^{jlk}
		2.5	16.2^{ij}	4.3^{ed}	4.8^{e}	50.6^{l}	-61.0^{jlk}
		1.25	16.2^{ij}	4.3^{ed}	4.8^{e}	50.6^{l}	-61.0^{jlk}
		0.625	13.2^{ih}	0.0^{d}	0.0^{ed}	50.6^{l}	-57.6^{mlk}
		Control	0.0^{dce}	0.0^{d}	0.0^{ed}	0.0^{b}	0.0^{wv}
		10	19.7^{gh}	7.4^{c}	22.6^{ed}	64.8^{k}	-84.1^{b}
	120	5	11.8^{de}	5.6^{bc}	18.9^{cd}	62.8^{kj}	-81.2^{cb}
		2.5	9.2^{dc}	5.6^{bc}	13.2^{b}	55.9^{ih}	-58.0^{fe}
		1.25	9.2^{dc}	5.6^{bc}	13.2^{b}	55.9^{gf}	-58.0^{fg}
		0.625	9.2^{dc}	5.6^{bc}	11.3^{b}	53.1^{de}	-34.8^{mlk}
		Control	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{tu}

Values in bold represents the MIC of plant extracts in mg/mL. Values within a column of treatment having the same superscript are not significantly different ($P < 0.05$) according to the LSD test. **WA:** *Wickerhamomyces anomalus*, **ZB1:** *Zygosaccharomyces bailii* CY0757, **ZB2:** *Zygosaccharomyces bailii* IGC4242, **RD:** *Rhodotorula dairenensis* and **YL:** *Yarrowia lipolytica*.

The methanol extract, showed the highest inhibitory activity (100 %) against *W. anomalus* at 5 mg/mL within the 48-hour period. But, as time progressed to 72 and 120 hours, the extract was seen to inhibit the yeast by 47.1 and 32.9 % respectively. This implied that the methanol extract was fungistatic at 5 mg/mL and fungicidal at 10 mg/mL against *W. anomalus*. Similarly, the methanol extract exerted 100 % inhibition against the CY0757 and IGC4242 strain of *Z. bailii* throughout the investigated time periods. However, after 120 hours the extract showed 73.6 % inhibition against the IGC4242 strain at 2.5 mg/mL, suggesting that the methanol extract was fungistatic at 2.5 mg/mL and fungicidal at 5 mg/mL against the IGC4242 strain of *Z. bailii*. The methanol extract further showed a concentration-dependent

inhibitory activity ranging from 32.8 - 56.9 % after 48 hours, 50.6 - 68.2 % after 72 hours and 50.3- 57.9 % after 120 hours and MIC of 0.625 mg/mL against *R. dairenensis*. Bioactivity against *R. dairenensis* at 48 and 72 hours was not significantly different. Interestingly, *Y. lipolytica* thrived in the presence of the methanol extract as time and concentration increased.

The acetone extract displayed less inhibitory activity compared to the methanol extract. This finding contradicts Dzoyem et al. (2016), who described acetone as the best solvent for extracting antimicrobial compounds from plants. The result from this study may be attributed to the 50 % concentration of acetone that was used to extract the plant material. As with the methanol extract, the acetone extract showed growth stimulatory activity on *Y. lipolytica* with increasing concentration and time. Although the yeast behaved slightly different toward the acetone extracts in the sense that it showed resistance at 5 mg/mL but as concentration decreased from 2.5 mg/mL the increase in yeast size became notable.

Liu et al. (2018) have reported that *M. oleifera* leaves are enriched with unsaturated fatty acids. This might explain the stimulatory effect exhibited by the extracts on *Y. lipolytica*, keeping in mind that the aerobic, dimorphic yeast utilizes hydrophobic substrates such as *n*-alkanes, fats, oils and fatty acids as carbon sources (Gonçalves et al., 2014). Nevertheless, further study is required to confirm the actual compound(s) in *M. oleifera* responsible for enhancing the growth of *Y. lipolytica;* a useful yeast in biofuel production (Darvishi et al., 2017).

Besides enhancing the growth of *Y. lipolytica* and showing inhibitory activity against *R. dairenensis*, the water extract was a poor growth inhibitor against the tested yeasts. This can be expected as organic solvents are more effective for extracting antimicrobial compounds from plants than water. Water being a polar solvent rarely shows antimicrobial activity (Dzoyem et al., 2016). Gull et al. (2016) proposed that this may be due to the unstable nature of bioactive compounds in water. Notwithstanding, the possibility of some plant water extracts exhibiting antimicrobial activity is not entirely ruled out, for example, Gonelimali et al. (2018) demonstrated that the water extracts of roselle and clove showed antimicrobial activity against some food pathogens and spoilage organisms. Gebreyohannes et al. (2019) also found that the hot water extract of wild mushrooms can inhibit the growth of *E.coli, C. parapsilosis, C. albicans, S. aureus* and *K. pneumonia*.

As depicted in Table 2, the mean values of growth inhibition (%) within a column of treatment were significantly different across the time intervals (48, 72 and 120 hours), signifying that the length of time the yeasts were exposed to the plant extracts influenced the level of anti-yeast or stimulatory activity. Also, there were significant differences between various concentrations of extracts used particularly against *Y. lipolytica, R. dairenensis, W. anomalus, Z. bailii* CY0757 and IGC4242. It was observed that the anti-yeast and stimulatory activity of the plant extracts increased as concentration increased to 10 mg/mL.

It is known that secondary metabolites from plant sources have broad spectrum activity against microorganisms (Hugo and Hugo, 2015; Kone et al., 2019). In that light, the variation observed in the yeasts sensitivity to the extracts may be ascribed to the presence of bioactive compounds in different concentrations and the solvents used for extraction, since the polarity of solvents is mainly accountable for the various degrees of active compounds extracted from plants (Wanyo et al., 2016; Tohma et al., 2019). Moreover, the solubility of the extracted anti-yeast compounds in 50 % methanol may justify the highest activity exhibited by the extract (Table 2), making 50 % methanol the best solvent for extracting anti-yeast compounds from *M. oleifera* compared to 50 % acetone or water.

CONCLUSION

Although the extracts of *M. oleifera* consist of various secondary metabolites with anti-yeast activities, they do not have the potential for use as natural preservatives in fruit juice due to their inability to inhibit the growth of certain yeasts associated with fruit juice spoilage. However, *M. oleifera* extracts may be used to stimulate the growth of *Yarrowia lipolytica* for use in biofuel production.

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Literature cited

Al Ghasham, A., Al Muzaini, M., Qureshi, K.A., Elhassan, G.O., Khan, R.A., Farhana, S.A., Hashmi, S., El-Agamy, E., and Abdallah, W.E. (2017). Phytochemical screening, antioxidant and antimicrobial activities of methanolic extract of *Ziziphus mauritiana* Lam. leaves collected from Unaizah, Saudi Arabia. Int. J. of Pharm Res. Allied Sci. *6(3)*, 33–46.

Almeida, E.T.C., de Souza, G.T., Guedes, J.P.S., Barbosa, I.M., de Sousa, C.P., Castellano, L.R.C., Magnani, M., and de Souza, E.L. (2019). *Mentha piperita* L. essential oil inactivates spoilage yeasts in fruit juices through the perturbation of different physiological functions in yeast cells. Food Microbiol. *82*, 20–29. https://doi.org10.1016/j.fm.2019.01.023

Bolarinwa, I.F., Aruna, T.E., and Raji, A.O. (2019). Nutritive value and acceptability of bread fortified with moringa seed powder. JSSAS. *18*, 195–200. https://doi.org/10.1016/j.jssas.2017.05.002

Bukvicki, D., Gottardi, D., Tyagi, A.K., Veljic, M., Marin, P.D., Vujisic, L., Guerzoni, M.E., and Vannini, L. (2014). *Scapania nemorea* liverwort extracts: investigation on volatile compounds, in vitro antimicrobial activity and control of *Saccharomyces cerevisiae* in fruit juice. LWT-Food Sci. Technol. *55*, 452–458. https//doi.org/10.1016/j.lwt.2013.09.029

Cardador, M.J. and Gallego, M. (2015). Haloacetic acids content of fruit juices and soft drinks. Food Chem. *173*, 685–693. https://doi.org/10.1016/j.foodchem.2014.10.105

Corbett, K.M. and de Smidt, O. (2019). Culture-dependent diversity profiling of spoilage yeasts species by PCR-RFLP comparative analysis. Food Sci Technol Int. *25*, 671–679. https://doi.org/10.1177/1082013219856779

Darvishi, F., Fathi, Z., Ariana, M., and Moradi, H. (2017). *Yarrowia lipolytica* as a workhorse for biofuel production. Biochem. Eng. J. *127*, 87–96. https://doi.org/10.1016/j.bej.2017.08.013

Djande, C.Y.H., Piater, L.A., Steenkamp, P.A., Madala, N.E., and Dubery, I.A. (2018). Differential extraction of phytochemicals from the multipurpose tree, *Moringa oleifera*, using green extraction solvents. S Afr. J. Bot. *115*, 81–89. https://doi.org/10.1016/j.sajb. 2018.01.009

Dzoyem, J.P., Aro, A.O., McGaw, L.J., and Eloff, J.N. (2016). Antimycobacterial activity against different pathogens and selectivity index of fourteen medicinal plants used in southern Africa to treat tuberculosis and respiratory ailments. S Afr. J. Bot. *102*, 70–74. https://doi.org/10.1016/j.sajb.2015.08.002

Gebreyohannes, G., Nyerere, A., Bii, C., and Sbhatu, D.B. (2019). Determination of antimicrobial activity of extracts of indigenous wild mushrooms against pathogenic organisms. Evid.-Based Complementary Altern. Med. 2019: 1–7. https://doi.org/10.1155/2019/6212673

Gonçalves, F.A.G., Colen, G., and Takahashi, J.A. (2014). *Yarrowia lipolytica* and its multiple applications in the biotechnological industry. Sci. World J. 2014, 1–14. https://doi.org/10.1155/2014/476207

Gonelimali, F.D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M., and Hatab, S.R. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. Front. Microbiol. *9*, 1639. https://doi.org/10.3389/fmicb.2018.01639

Guedes, J.P.S., Da Costa Medeiros, J.A., de Souza e Silva, R.S., de Sousa, J.M.B., da Conceição, M.L., and de Souza, E.L. (2016). The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices. Int. J. Food Microbiol. *238*, 183–192. https://doi.org/10.1016/j.ijfoodmicro.2016.09.005

Gull, I., Javed, A., Aslam, M.S., Mushtaq, R., and Athar, M.A. (2016). Use of *Moringa oleifera* flower pod extract as natural preservative and development of SCAR marker for its DNA based identification. Biomed Res. Int. 2016, 1–12. https://doi.org/10.1155/2016/7584318

Haneen, H.S.M. (2015). Effect of dried *Moringa oleifera* leaves on the nutritional and organoleptic characteristics of cookies. Alexandria Sci. Exchange J. *36(4),* 297–305. https://doi.org/10.21608/asejaiqjsae.2015.2934

Hugo, C.J. and Hugo, A. (2015). Current trends in natural preservatives for fresh sausage products. Trends Food Sci. Tech. *45*, 12–23. https://doi.org/10.1016/j.tifs.2015.05.003

Jayawardana, B.C., Liyanage, R., Lalantha, N., Iddamalgoda, S., and Weththasinghe, P. (2015). Antioxidant and antimicrobial activity of drumstick (*Moringa oleifera*) leaves in herbal chicken sausages. LWT-Food Sci. Technol. *64*, 1204–1208. https://doi.org/10.1016/j.lwt.2015.07.028

Kone, A.P., Desjardins, Y., Gosselin, A., Cinq-Mars, D., Guay, F., and Saucier, L. (2019). Plant extracts and essential oil product as feed additives to control rabbit meat microbial quality. Meat Sci. *150*, 111–121. https://doi.org/10.1016/j.meatsci.2018.12.013

Kumar, S., Yadav, A., Yadav, M., and Yadav, J.P. (2017). Effect of climate change on phytochemical diversity, total phenolic content and in vitro antioxidant activity of *Aloe vera* (L.) Burm.f. BMC Res. Notes. *10(1),* 60. https://doi.org/10.1186/s13104-017-2385-3

Liu, Y., Wang, X.Y., Wei, X.M., Gao, Z.T., and Han, J.P. (2018). Values, properties and utility of different parts of *Moringa oleifera*: an overview. *Chinese Herbal Medicines. 000*, 1–8.

López-Carballo, G., Gómez-Estaca, J., Catalá, R., Hernández-Muñoz, P., and Gavara, R. (2012). Active antimicrobial food and beverage packaging. In: Emerging Food Packaging Technologies*: Principles and Practice*. Yam, K.L. and Lee, D.S., Eds. València, Spain: Woodhead Publishing Series in Food Science, Technology and Nutrition. pp. 27–54. https://doi.org/10.1533/9780857095664.1.27

Maqsood, M., Qureshi, R., Arshad, M., Ahmed, M.S., and Ikram, M. (2017). Preliminary phytochemical screening, antifungal and cytotoxic activities of leaves extract of *Moringa oleifera* lam. from salt range, Pakistan. Pak. J. of Bot. *49(1)*: 353–359.

Ng, K.R., Lyu, X., Mark, R., and Chen, W.N. (2019). Antimicrobial and antioxidant activities of phenolic metabolites from flavonoid-producing yeast: potential as natural food preservatives. Food Chem. *270*, 123–129. https://doi.org/10.1016/j.foodchem.2018.07.077

Ngo, T.V., Scarlett, C.J., Bowyer, M.C., Ngo, P.D., and Vuong, Q.V. (2017). Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of Salacia chinensis L. J. Food Qual. *2017,* 8. https://doi.org/10.1155/2017/9305047

Padmalochana, K. (2018). Anti-inflammatory activity and phytochemical analysis of *Moringa oleifera* ethanol and acetone leaves extract. J. Drug Deliv. Ther. *8(6-s),* 269–273.

Patel, N., Patel, P., Patel, D., Desai, S., and Meshram, D. (2014). Phytochemical analysis and antibacterial activity of *Moringa oleifera.* Int. J. Med. and Pharm. Sci. *4(2),* 27–34.

Shanmugavel, G., Prabakaran, K., and George, B. (2018). Evaluation of phytochemical constituents of *Moringa oleifera* (Lam.) leaves collected from Puducherry region, South India. Int. J. Zool. Appl. Biosci. *3(1),* 1–8.

Soto, K.M., Iturriaga, M.H., Piña, G.L., Bárcenas, G.L., and Mendoza, S. (2019). Antimicrobial effect of nisin electrospun amaranth: pullulan nanofibers in apple juice and fresh cheese. Int. J. Food Microbiol. *295*, 25–32. https://doi.org/10.1016/j.ijfoodmicro.2019.02.001

Tohma, H., Altay, A., Köksal, E., Gören, A.C., and Gülçin, I. (2019). Measurement of anticancer, antidiabetic and anticholinergic properties of sumac (*Rhus coriaria*): analysis of its phenolic compounds by LC–MS/MS. J. Food Meas. Charact. *13(2),* 1607–1619. https://doi.org/10.1007/s11694-019-00077-9

Vinoth, B., Manivasagaperumal, R., and Balamurugan, S. (2012). Phytochemical analysis and antibacterial activity of *Moringa oleifera* lam. Int. J. Res. Biol. Sci. *2(3),* 98–102.

Wanyo, P., Kaewseejan, N., Meeso, N., and Siriamornpun, S. (2016). Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by-products. Appl. Biol. Chem. *59(3),* 373–384. https://doi.org/10.1007/s13765-016-0173-8